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In vivo assessment of toxicity and pharmacokinetics of methylglyoxal Augmentation of the curative effect of methylglyoxal on cancer-bearing mice by ascorbic acid and creatine

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Abstract

Previous in vivo studies from several laboratories had shown remarkable curative effect of methylglyoxal on cancer-bearing animals. In contrast, most of the recent in vitro studies have assigned a toxic role for methylglyoxal. The present study was initiated with the objective to resolve whether methylglyoxal is truly toxic in vivo and to reassess its therapeutic potential. Four species of animals, both rodent and non-rodent, were treated with different doses of methylglyoxal through oral, subcutaneous and intravenous routes. Acute (treatment for only 1 day) toxicity tests had been done with mouse and rat. These animals received 2, 1 and 0.3 g of methylglyoxal/kg of body weight in a day through oral, subcutaneous and intravenous routes respectively. Chronic (treatment for around a month) toxicity test had been done with mouse, rat, rabbit and dog. Mouse, rat and dog received 1, 0.3 and 0.1 g of methylglyoxal/kg of body weight in a day through oral, subcutaneous and intravenous routes respectively. Rabbit received 0.55, 0.3 and 0.1 g of methylglyoxal/kg of body weight in a day through oral, subcutaneous and intravenous routes respectively. It had been observed that methylglyoxal had no deleterious effect on the physical and behavioral pattern of the treated animals. Fertility and teratogenecity studies were done with rats that were subjected to chronic toxicity tests. It had been observed that these animals produced healthy litters indicating no damage of the reproductive systems as well as no deleterious effect on the offspring. Studies on several biochemical and hematological parameters of methylglyoxal-treated rats and dogs and histological studies of several organs of methylglyoxal-treated mouse were performed. These studies indicated that methylglyoxal had no apparent deleterious effect on some vital organs of these animals. A detailed pharmacokinetic study was done with mouse after oral administration of methylglyoxal. The effect of methylglyoxal alone and in combination with creatine and ascorbic acid on cancer-bearing animals had been investigated by measuring the increase in life span and tumor cell growth inhibition. The results indicated that anticancer effect of methylglyoxal was significantly augmented by ascorbic acid and further augmented by ascorbic acid and creatine. Nearly 80% of the animals treated with methylglyoxal plus ascorbic acid plus creatine were completely cured and devoid of any malignant cells within the peritoneal cavity.

Keywords: Methylglyoxal; Ascorbic acid; Creatine; Toxicity; Pharmacokinetics; Cancer treatment

Introduction

As early as 1913, it was observed that methylglyoxal is converted to D-lactic by a strong and ubiquitous enzyme system. But how methylglyoxal is formed in organisms and from what precursor were unknown at that time. However, in 1970s and 1980s, the metabolic pathway for methylglyoxal in different organisms had been established with the isolation, purification and characterization of several enzymes responsible for the formation and breakdown of methylglyoxal. That methylglyoxal is a normal metabolite has been firmly established (for a review, see Ray and Ray, 1998).

The anticancer property of methylglyoxal was also known for a long time. In the early 1960s, Szent-Györgyi et al. proposed that methylglyoxal is a natural growth regulator and

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can act as an anticancer agent (Együd and Szent-Györgyi, 1966, Együd and Szent-Gtörgyi, 1968; Szent-Györgyi et al., 1967; Szent-Györgyi, 1979). They also provided strong experimental evidence in support of the hypothesis. When mice were inoculated with ascites sarcoma 180 cells and then treated with methylglyoxal, no tumor developed (Együd and Szent-Gtörgyi, 1968). At the same time, Apple and Greenberg (1967, 1968) showed remarkable curative effect of methylglyoxal in experiments with mice bearing a wide variety of cancers. Other investigators (Conroy, 1979; Elvin and Slater, 1981) had also observed similar anticancer effect of methylglyoxal.

Együd and Szent-Györgyi (1966) suggested that the anticancer property of methylglyoxal is mediated through the growth inhibitory effect of methylglyoxal, which in turn is due to the inhibition of protein synthesis by methylglyoxal. However, whether there is a qualitative difference in the effect of methylglyoxal between normal and malignant cells had not been systematically investigated. Moreover, very little studies had been done previously with human tissue materials.

Subsequent studies had indicated that methylglyoxal is tumoricidal. It inhibits both glycolysis and mitochondrial respiration of specifically malignant cells (Ray et al., 1991; Halder et al., 1993; Biswas et al., 1997). With a wide variety of post-operative human tissues as also animal tissues and cells, both normal and malignant, it had been observed that methylglyoxal inhibits mitochondrial respiration (at the level of complex I) and inactivates glyceraldehyde-3-phosphate dehydrogenase of specifically malignant cells (Halder et al., 1993; Ray et al., 1994, 1997a, 1997b; Biswas et al., 1997). These results strongly suggest that these two enzymes are altered specifically in malignant cells.

In contrast to the positive effect of methylglyoxal as referred to above, recent publications on methylglyoxal research overwhelmingly state that methylglyoxal is toxic. Numerous papers have appeared in the literature, which mostly with in vitro studies have shown that methylglyoxal reacts with arginine, lysine and free terminal amino groups in proteins resulting in AGE (advanced glycation end products) formation. The possibilities of many deleterious effects of methylglyoxal in the body have been extrapolated based mostly on these in vitro studies. The notable complications are related to diabetes and cataract formation (Thornalley, 1996; Lee et al., 1999; Morgan et al., 2002; Roberts et al., 2003). Evidence had also been put forward that methylglyoxal is mutagenic (Murata-Kamiya et al., 2000) and induces reactive oxygen species formation (Chan et al., 2005; Chang et al., 2005). Since relatively little in vivo studies with methylglyoxal have been done, it is logical to conceive that many of the purported in vitro toxic effects of methylglyoxal may be overwhelmed by the many countervailing reactions in an intact animal. This consideration especially stems from the reports of significant curative effect of methylglyoxal towards cancer-bearing animals that had been observed and mentioned above. Moreover, in vitro studies with human samples had indicated the inhibitory effect of methylglyoxal on glyceraldehyde-3-phosphate dehydrogenase and mitochondrial complex I of specifically malignant cells. The results of all these studies strongly demand that methylglyoxal alone or in combination with other substances should be tested for the possible efficacy of treating cancer patients. However, it has not been tested until recent past. On the other hand, methylglyoxal bis-guanylhydrazone, a derivative of methylglyoxal, had undergone clinical trial with limited success (Dunzendorfer et al., 1986; Friedman et al., 1986; Gastaut et al., 1987).

Before initiating any study with human subjects, it is imperative that a thorough study with animals for any toxic effect of the potential drug be tested. With this objective in mind and to understand the in vivo toxic effect of methylglyoxal in general, the present study was undertaken with both rodent and non-rodent species. Both acute and chronic toxicity studies as well as pharmacokinetic studies and analyses were made. Moreover, the curative effect of methylglyoxal in combination with other naturally occurring substances, ascorbic acid and creatine, has been further assessed. Although our studies are limited, the results do indicate that at the dose level studied methylglyoxal has apparently been devoid of any major toxic effect in vivo. This paper also reiterates the curative effect of methylglyoxal.

Materials and methods

Materials

Animals

Swiss albino mice and Sprague–Dawley rats of both sexes were used. Rabbits and dogs/bitches were of locally bred. Mice and rats received normal laboratory diet. Rabbits received gram, cabbage, carrot and lettuce and a mixture of vitamins. Dogs and bitches received standard healthy foods. All the animals received water ad libitum and were housed in rooms, temperature and humidity of which were maintained at 25–30 °C and 55–60% respectively.

The Institute's animal ethics committee approved the animal maintenance facility and the protocol of the experiments.

Chemicals

Methylglyoxal and creatine were obtained from Sigma Chemical Company, St. Louis, MO. Ascorbic acid was from S R L Mumbai, India.

Metabolite and enzyme assay kit

Hemoglobin assay kit was obtained from Monozyme India Limited, Hyderabad, India. Urea, glucose, creatinine, alkaline phosphatase, serum aspartate transaminase and serum alanine transaminase assay kits were obtained from Span Diagnostics Limited, Surat, India. Creatine kinase and creatine kinase MB assay kit were obtained from Bayer

Diagnostics India. The assays were done according to the manufacturers' instruction. These standard methods are widely used. For the sake of brevity, the methods are described briefly.

Methods

Metabolite estimation and enzyme assay

Hemoglobin was estimated by reacting the blood sample with Drabkin's reagent to convert it to cyanmethemoglobin and measuring the absorbance at 540 nm. Urea was estimated by reacting it with hot acidic diacetylmonoxime in the presence of semicarbarzide and measuring the rose-purple color at 525 nm. Glucose was estimated by glucose oxidase method in the presence of peroxidase and measuring the absorbance of the colored complex of hydroxybenzoate and 4-aminophenazone at 510 nm. Creatinine was measured by reacting it with alkaline picrate and measuring the absorbance of the red-colored complex at 520 nm. Alkaline phosphatase was assayed by its ability to convert phenyl phosphate to inorganic phosphate and phenol. The later reacts with 4-aminoantipyrine to form an orange-red-colored complex, which was measured at 510 nm. Aspartate transaminase was measured by reacting the reaction product oxaloacetate (from the substrate L-aspartic acid) with 2,4-dinitrophenyl hydrazine. The hydrazone thus formed gives a characteristic brown color when placed in an alkaline medium and was measured at 505 nm. Alanine transaminase was assayed similarly by 2,4-dinitrophenylhydrazine-alkali color reaction; the substrate and products are L-alanine and pyruvate respectively. Creatine kinase was assayed by monitoring the formation of ultimate reaction product NADPH from creatine phosphate and ADP in the presence of glucose, hexokinase, glucose 6-phosphate dehydrogenase and NADP. The reaction was monitored at 340 nm. The isozyme creatine kinase MB was assayed in a similar fashion but in the presence of the antibody to creatine kinase-M monomer.

Methylglyoxal estimation

Methylglyoxal was estimated by derivatization of methylglyoxal with 1,2-diaminobenzene to produce 2-methylquinoxaline according to the method of Cordeiro and Freire (1996) with some modifications.

Whole blood from either rat or mice was taken by heart puncture. To 1 ml of blood sample, 1 ml of distilled water was added and mixed thoroughly and then treated with 50 μl of 70% ice-cold perchloric acid and kept at room temperature for 30 min. It was then centrifuged at 8000 × *g* for 20 min at 4 °C. After rejecting the precipitate, the supernatant was brought to pH 7.0 by drop wise addition of saturated potassium carbonate solution. After 10 min, it was centrifuged at 8000 × *g* for 10 min at 4 °C. To 1 ml of the resulting supernatant was added 200 μl of 5 M perchloric acid, 500 μl of diaminobenzene in water, and the volume was made up to 2 ml with water. It was scanned in a spectrophotometer in wavelengths 200–400 nm. Maximum absorbance was observed in wavelength of 334 nm.

A standard solution of methylglyoxal instead of blood treated under identical conditions was scanned as above. The value at a particular concentration of methylglyoxal was used to calculate the amount of methylglyoxal present in the blood.

The authenticity of the method was confirmed further in blood sample by utilizing methylglyoxal in presence of glyoxalase I and GSH (Cooper, 1975), where no detectable 2-methylquinoxaline was formed (details not presented). The lowest amount of methylglyoxal that could be detected in our experimental set up was 1 nmol, and the recovery of methylglyoxal from test samples was approximately 95%.

For all toxicity and treatment studies, the amount of methylglyoxal applied was per kg body weight of the animal

Acute toxicity study. All the animals received methylglyoxal in two divided doses for only 1 day. Three modes of treatment were used: a) oral, through gastric cannula; b) subcutaneous and c) intravenous, through tail vein. For oral treatment, methylglyoxal was diluted in distilled water, and each mouse received 0.35–0.5 ml in a single dose. For subcutaneous and intravenous injections, methylglyoxal was diluted in normal saline. Moreover, for intravenous treatment, methylglyoxal solution was passed through a membrane filter of 0.2-μm pore size. For subcutaneous and intravenous injections, 0.25 ml or 0.15 ml of the solution was injected per dose respectively. For test with mice, the animals were divided in groups each containing 6 animals either male or female weighing 18–20 g. For rats, the animals were divided in groups each containing 5 animals either male or female weighing 80–100 g. The control group in each mode of treatment received either water or normal saline.

Long-term (chronic) toxicity study. Mode of treatment and dilution by either water or normal saline were identical to acute toxicity study. Chronic toxicity study was made with four species of animals: mouse, rat, rabbit and dog.

Mouse. For experiment with these animals, 4 batches of mice were used per mode of treatment. Each batch contained 6 animals either male or female weighing 18–25 g. All the animals received methylglyoxal in two divided doses per day for a total period of 6 weeks; for oral and subcutaneous studies, 6 days per week, for intravenous, 4 days per week due to swelling of tail and adjoining areas. For oral, subcutaneous and intravenous administrations, each animal received 0.7, 0.2 and 0.25 ml per dose respectively. In all the cases, control group received water or normal saline in respective manner.

Histological studies were done with mice, which had received methylglyoxal orally once a day for 10 weeks, 7 days per week. After end of the treatment, the mice were killed, and several organs were excised and processed for

histological studies. Bone marrow cells were taken from the marrow cavity of the femur bone.

Rat. For experiment with these animals, 4 batches of rats were used per mode of treatment. Each batch contained 5 rats either male or female weighing 75–80 g per animal. For oral treatment, each rat received 1.5 ml of methylglyoxal solution once a day for 6 weeks, 6 days per week. For subcutaneous treatment, each rat received 0.9 ml of methylglyoxal solution once a day for 4 days per week for 4 weeks. Then, the rats were injected for 3 cycles; one cycle consisted of injections for two consecutive days followed by a rest of 1 day. In intravenous treatment, each rat received 0.5 ml of methylglyoxal per dose, once a day for 6 weeks, 6 days per week.

Rabbit. For experiments with rabbits, two groups were used per mode of treatment. Each group in each mode of treatment consists of 4 animals, either male or female. However, each animal was placed in a separate cage. For oral treatment, each rabbit received 12–15 ml of methylglyoxal solution once a day for 6 weeks, 6 days per week. For subcutaneous treatment, each animal received 2–2.5 ml of methylglyoxal once a day for 6 weeks, 4 days per week. For intravenous treatment, each rabbit received 1.4–1.6 ml once a day for 3 weeks, 4 days per week. Then, the animals received injections for 2 consecutive days followed by a rest of 1 day for total period of 15 days.

Long-term toxicity test with non-rodents (dog and bitch). Total of 6 animals (4 dogs and 2 bitches) were used for this experiment. The animals were 3–4 months old. Three different modes of treatment were used; two animals received the formulation orally, the other two by intravenous injection and the other two by subcutaneous injection. The total period of treatment was for 4 weeks (5 days per week). For oral treatment, the animals were fed 10 ml solution of methylglyoxal once a day. For subcutaneous and intravenous treatments, the animals were injected 1.5–2.0 ml of methylglyoxal solution once a day.

Pharmacokinetic studies

Single dose study. In single dose study, a batch of 52 mice received methylglyoxal dissolved in water as a single oral dose of either 0 or 50 or 100 or 200 mg/kg of body weight. For methylglyoxal estimation, blood samples were collected by heart puncture, at 0 h (predose) and at intervals of 1 h up to 6 h and then at an interval of 2 h up to 12 h. Two animals were sacrificed for each dose or no dose, and the blood was pooled, and methylglyoxal was estimated. The entire set of abovementioned experiment was repeated 6 times.

Different pharmacokinetic parameters were determined with a one-compartment model with lag time and first order absorption and elimination. Data from single dose experiments were used to set dose for repeat dose experiments (Benet et al., 1996).

We had observed that the concentrations of methylglyoxal in both plasma and whole blood were almost identical in mice. These mice were both untreated and orally treated with methylglyoxal. So, in all our experiments, we measured the level of methylglyoxal in whole blood.

Repeat dose study. For this experiment, 46 mice in each group received orally 100 mg of methylglyoxal per kg of body weight/day in two divided doses (8 am and 8 pm). At day 0, at 10 am, besides 46 mice, 2 mice that did not receive any methylglyoxal were sacrificed and blood was collected by heart puncture, pooled and methylglyoxal was estimated.

From 46 mice that had started orally receiving methylglyoxal, two mice on each day were sacrificed and methylglyoxal estimated in a similar fashion. The administration of methylglyoxal and sacrifice of animals for methylglyoxal estimation continued on days 1–9 (each day) and on days 12, 15, 19, 22, 25, 28, 29 and 30. After that, methylglyoxal administration was discontinued, but two animals from the remaining animals were sacrificed on each day for methylglyoxal estimation (days 31–36).

Table 1 presents a summary protocol of mode and dose of treatment of methylglyoxal for different studies. It appears that there are some variations in the doses applied to different animals through different routes for different studies. But, in all the cases, the doses that were administered were significantly higher than the intended dose for treatment that had been worked out from the previous works of Együd and Szent-Görgyi (1968) and Apple and Greenberg (1968) and also the study presented in this paper.

Biochemical analyses of blood

Rat. One male and one female from each group (i.e. oral, intravenous and subcutaneous and control) on which long-term toxicity tests were performed were chosen at random 1 week after completion of the treatment, and blood was extracted, and 2.5–3.0 ml was pooled from each group. After coagulation, the sera was separated by centrifugation at 2000 rpm for 5 min. Hemoglobin was also measured from a small sample of uncoagulated blood.

Dog. For biochemical analysis, blood samples from each individual animal were collected and processed similarly to that of rats. The samples were collected just before the treatment commenced, in mid-phase of the treatment and 7 days after completion of the treatment. The samples were analyzed for the activities of several enzymes and metabolite contents as per the methods described in the respective assay kit and are briefly mentioned before. Hemoglobin estimation and cell count were done with an uncoagulated sample.

In vivo testing of the efficacy of methylglyoxal, ascorbic acid and creatine

Increase in life span study. For increase in life span study (ILS), testing was evaluated by calculating the median survival time (MST) of the treated (T) and control

Table 1
A summary protocol of mode and dose of treatment of methylglyoxal for different studies

Animal and different studies	Mode and dose (in gm/Kg of body weight) of treatment		
	Oral	Subcutaneous	Intravenous
Mouse			
Single for toxicity study ($n^a = 6 \times 8$)	2	1	0.3
Single for pharmacokinetic study ($n = 52 \times 6$)	0.2	–	–
Multiple for toxicity study ($n = 6 \times 4$)	1	0.3	0.1
Multiple for pharmacokinetic study ($n = 46 \times 3$)	0.1	–	–
Multiple for histological study ($n = 20 \times 2$)	0.5	–	–
Rat			
Single for toxicity study ($n = 5 \times 4$)	2	1	0.3
Multiple for toxicity study ($n = 5 \times 4$)	1	0.3	0.1
Multiple for biochemical study	1	–	–
Rabbit			
Multiple for toxicity study ($n = 4 \times 2$)	0.55	0.3	0.1
Dog and bitch			
Multiple ($n = 6 \times 1$)	1	0.3	0.1

Duration of the treatment is described in the text.
[a] n = number of animals in each group × number of groups.

(C) groups and expressed as ILS value [$(T / C - 1) \times 100$]. The ILS value of >25 is considered for significant activity in these tumors (Geran et al., 1972; Sanyal et al., 1993).

Increase in body weight. For this experiment, mice were weighed periodically during and after the therapy. The results are expressed as percentage increase in the body weight

using the following relationship : percent increase

$$= \frac{\text{average increase in body weight}}{\text{average initial body weight}} \times 100$$

Tumor growth inhibition study. For this study, the total number of EAC cells was counted. The ascites fluid containing cells were quantitatively removed from peritoneal cavity of two mice for a particular drug combination. The cavity was further washed twice with a fixed volume of 0.9% NaCl. The washing and the ascites fluid containing the cells were pooled and centrifuged at 2000 × g for 5 min. The packed cell volumes were noted. A fixed amount of aliquot from the packed cells was appropriately diluted, and the number of cells was counted in a hemocytometer. Averages ($\bar{X} \pm$ SEM) were made of these two parameters, and percentage inhibitions [$(1 - T / C) \times 100$] were calculated for each dose of different test combinations.

Statistical analysis. Values were recorded as mean ± SEM. Experimental results were analyzed by Student's t test. $P < 0.05$ was considered as the level of significance for values obtained for treated compound to control.

Results

Toxicity study in animals

Acute toxicity study

Acute toxicity study was done with two species of animals, mouse and rat. The maximum dose of methylglyoxal for each mouse was for oral 2 g, for subcutaneous 1 g and for intravenous 0.3 g.

Table 2
Long-term (chronic) toxicity in animals: measurement of body weight

Animal	Weight of animals[a]					
	Oral		Subcutaneous		Intravenous	
	Control	Treated	Control	Treated	Control	Treated
Mice						
Day 1	21.16 ± 1.06	20.6 ± 1.1	18.16 ± 1.34	22.16 ± 0.68	23.16 ± 3.2	23.3 ± 2.13
Day 90	23.6 ± 0.74	22.3 ± 1.79	18.8 ± 0.68	22.87 ± 0.63	25.4 ± 2.32	25.3 ± 0.74
Rat						
Day 1	78.6 ± 2.15	79.4 ± 0.48	78.4 ± 2.4	79.6 ± 2.05	79.5 ± 2.29	79.1 ± 1.34
Day 60	80.3 ± 1.7	81.9 ± 0.89	81.2 ± 2.05	80 ± 1.26	82.3 ± 1.59	82.9 ± 1.4
Rabbit						
Day 1	1.4 ± 0.07	1.48 ± 0.05	1.12 ± 0.08	1012 ± 0.08	1.5 ± 0.09	1.45 ± 0.08
Day 60	1.57 ± 0.08	1.61 ± 0.05	1.3 ± 0.07	1.32 ± 0.04	1.61 ± 0.08	1.55 ± 0.09

Amount of methylglyoxal received by each animal: mouse and rat- 1gm (oral), 0.3 gm (subcutaneous) and 0.1 gm (intravenous); rabbit –0.55 gm (oral), 0.3 gm (subcutaneous) and 0.1 gm (intravenous). Total number of animals in each group including control and different modes of treatment: 6 (mouse), 5 (rat) and 4 (rabbit). Each set of experiment was repeated 4 times for mouse and rat and for rabbit 2 times respectively. For each mode of treatment a similar study with lesser amount of methylglyoxal was done for mouse and rat and similar results were obtained.
[a] Weight of animals for mouse and rat in gm and for rabbit in Kg.

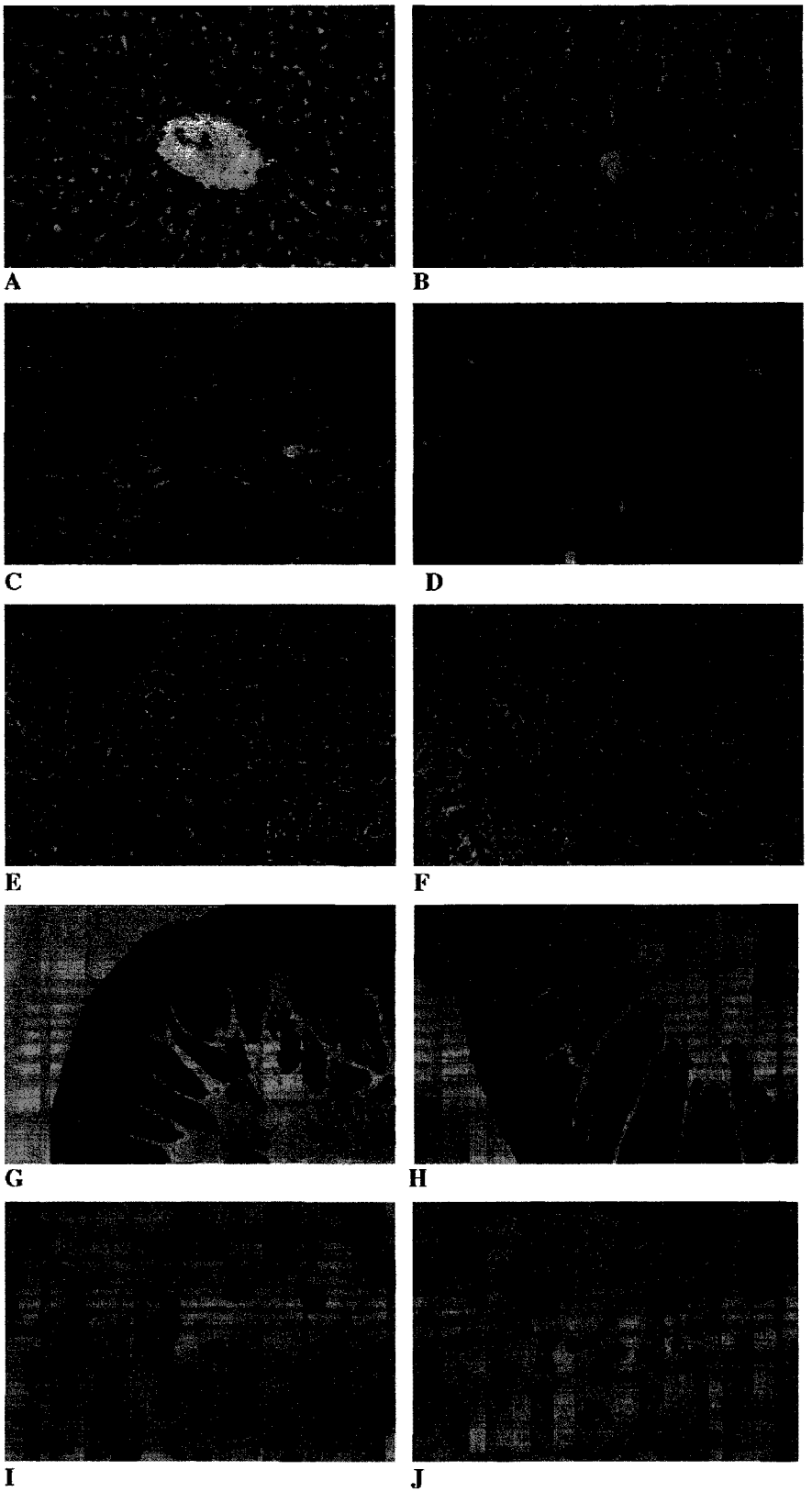

Fig. 1. Histological examination of different organs of mouse, both untreated (control) and treated orally by methylglyoxal. (A and B) Liver, control and treated respectively; magnification 40×. (C and D) Kidney, control and treated respectively; magnification 10×. (E and F) Spleen, control and treated respectively; magnification 40×. (G and H) Duodenum, control and treated respectively; magnification 10×. (I and J) Bone marrow, control and treated respectively; magnification 100×. The stain used for bone marrow was Leishmann, for other organs, hematoxylin and eosin.

Table 3
Biochemical tests of blood/sera of rats

Test	Hb, metabolite content and marker enzyme activities			
	Control	Oral	Intravenous	Subcutaneous
Hb (gm/dl)	10.65 ± 0.85	10.4 ± 0.3	10.2 ± 0.8	10.35 ± 0.35
Serum glucose (mg/dl)	115 ± 5	109 ± 6	102 ± 10	122 ± 3
Serum Urea (mg/dl)	25.4 ± 1.8	24.3 ± 1.9	26.3 ± 2.1	23.5 ± 1.3
Serum creatinine (mg/dl)	0.89 ± 0.15	0.82 ± 0.03	0.90 ± 0.02	0.85 ± 0.03
Serum aspartate transaminase (units/ml)	155 ± 6.5	132 ± 8	125 ± 5	141 ± 7
Serum alanine transaminase (units/ml)	32 ± 2.4	24 ± 2.5	28 ± 1.6	28 ± 2.8
Serum alkaline phosphatase (KA units)	42.6 ± 1.2	39.3 ± 1.6	41.6 ± 1.2	45.3 ± 1.8
Creatine kinase (units/ml)	0.46 ± 0.03	0.37 ± 0.05	0.36 ± 0.04	N.D.
Creatine kinase-MB (units/ml)	0.22 ± 0.03	0.16 ± 0.03	0.18 ± 0.01	N.D.

All the animals were observed up to 90 days. No death was observed. All the animals remained healthy, no weight loss and behavioral change were observed. No external toxic symptoms were noted in animals in general appearance and in respect of skin and hair texture and in behavioral pattern in respect of food and water intake and activity. No other abnormalities were found. We could not determine the LD_{50} because the abovementioned high dose of treatment has no apparent effect on the animals. Acute toxicity study with rat was done in a similar fashion, and similar results were obtained (details of the results are not presented).

Long-term (multiple dose) toxicity study

Long-term toxicity study was done with four species of animals: mouse, rat, rabbit and dog. Mortality, general physical and behavioral conditions and changes of body weight if any were observed for the four different species of animals. Besides observing these parameters, biochemical tests were also performed in blood samples of dog and rat. Fertility and teratogenecity studies were performed with rats and mice. Histological studies were done with several organs of rat subjected to methylglyoxal treatment and compared with that of the untreated animals.

Long-term toxicity (multiple dose) test with mice

All the animals were observed up to 90 days after completion of the treatment and were found to remain healthy. No death and toxic effect (physical and behavioral) were observed during the observation period. However, for subcutaneous treatment, swelling and damage of hair at the point of injection were noted for control and treatment groups. Results (changes in body weight) are presented in Table 2.

Histological studies with mouse

Histological studies were done with several organs of mouse, and the results are presented in Fig. 1. These mice received methylglyoxal orally once a day for 10 weeks, 7 days per week. It appears from the figure that none of the

Table 4
Effect of methylglyoxal treatment on the level of several marker enzymes and metabolites of sera and on cell population of blood of dog and bitch

Test	Activities of enzymes, metabolite concentration and blood cells								
	Before treatment			Mid-phase treatment			After treatment		
	A	B	C	A	B	C	A	B	C
Serum glucose (mg/dl)	99	81	87	102	96	96	85	75	82
Serum urea (mg/dl)	14	10	12	14	17	14	16	17	17
Serum alanine transferase (IU/L)	16	42	16	28	32	20	32	30	16
Serum aspartate transferase (IU/L)	14	29	22	20	19	14	22	23	21
Serum Alkaline phosphatase (IU/L)	192	269	317	442	190	379	185	179	193
Haemoglobin (gm/c.mm)	7.3	9.2	9.5	9.2	9.5	10.0	8,4	6.6	8.4
R.B.C. (per c.mm)	n.d.	n.d.	n.d.	3,500,000	3,550,000	3,600,000	3,400,000	2,800,000	3,420,000
W.B.C. (per c.mm)	n.d.	n.d.	n.d.	6700	9700	7800	7800	11400	9400
Neutrophils (%)	n.d.	n.d.	n.d.	50	58	53	69	49	67
Lymphocytes (%)	n.d.	n.d.	n.d.	45	30	44	26	44	27
Monocytes (%)	n.d.	n.d.	n.d.	3	2	2	2	3	2
Eosinophils (%)	n.d.	n.d.	n.d.	2	10	1	3	4	4
Basophils (%)	n.d.	n.d.	n.d.	0	0	0	0	0	0
				Mild hypochromia. No abnormal cells.			Mild hypochromia. No abnormal cells.		
Weight (in kg)	3	4.5	2.5	n.d.	n.d.	n.d.	4.5	6	3.8

(A – dog, B – bitch, C – dog).
n.d. : not determined.

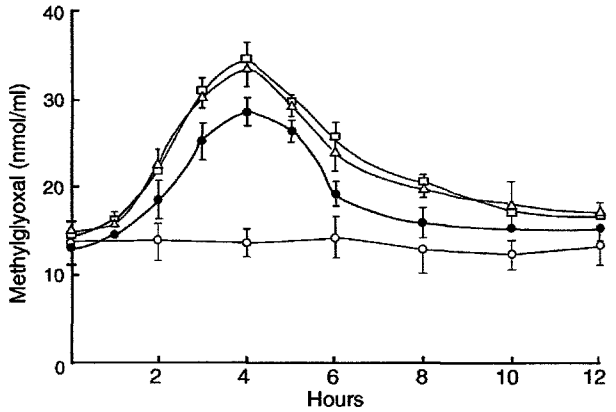

Fig. 2. .Blood methylglyoxal concentrations in mouse after single oral dose of methylglyoxal. ○, ●, Δ and □ represent 0, 50, 100 and 200 mg of methylglyoxal respectively.

organs tested by histological examination had any adverse effect on oral treatment of methylglyoxal at the particular dose level.

Long-term toxicity (multiple dose) tests with rat

All the animals except those used for biochemical studies (see below) were observed up to 90 days after completion of the treatment and were found to remain healthy. No toxic effect on physical condition and behavioral pattern such as hair texture, food intake etc. and death were observed. However, the subcutaneous injections appeared to be painful for both treated and control groups. The pain appeared to persist for several minutes after injection. In the animals, which received intravenous injections, swelling appeared in the tail and adjoining regions from 3rd week of the treatment. The swelling remained up to about 10 days from end of the treatment. Details are described in Table 2.

Long-term toxicity studies with rabbit and dog

We also investigated the long-term effect of methylglyoxal treatment of two other species, rabbit and dog. We observed general physical conditions and behavioral pattern of these treated animals with that of the control animals. Similar to the findings of long-term tests on mouse and rat, the treated animal (both rabbit and dog) showed no abnormalities in comparison to the control group of animals. We also measured the body weights of the animals. Table 2 shows the body weight of rabbit up to 60 days of observation period. Besides, we also measured several marker enzymes and metabolites in the blood and sera of dog (see below).

Reproductive and teratogenic studies on rat

Because our formulation is intended basically for the treatment of cancer patients, in our opinion, reproductive study is not much relevant. However, in the course of toxicity studies with mouse and rat, we kept some male and female animals in a single cage. Some of the female animals after completion of the treatment during the observation period gave birth to healthy litters. So, we tested whether methylglyoxal had any adverse effect on the fertility and teratogenicity.

Fertility. As methylglyoxal had been found to have no adverse effect, we performed fertility tests. For this, 4 female and 1 male were kept together, and 3 such groups were given the formulation orally. Similarly, 3 groups received the formulation as intravenous injections. The dose and treatment schedules were similar to that for the chronic toxicity test. Each female animal was pregnant and on an average gave birth to 5 healthy litters. Neither deformation of organs nor any other abnormalities were observed among the litters.

Teratogenicity. For this test, mating was performed between mice as mentioned above in the case of fertility studies. However, the female animals did not receive any treatment until they were pregnant. But, as soon as they conceive, as indicated by vaginal plug formation, the treatment (oral and intravenous) started and continued for 3 weeks in a similar fashion for chronic toxicity studies. In this experiment also, the female mice gave birth to healthy litters. These litters also grew up healthy with no signs of abnormality. Healthy litters were born again when mating was performed among these animals.

Effect on several marker enzymes and metabolites of blood/sera of rats and dogs, which were subjected to long-term toxicity tests

As mentioned before, we had observed in acute toxicity studies with mouse and rat and in chronic toxicity studies with mouse, rat, rabbit and dog that there was no apparent toxic effect of methylglyoxal in physical condition and

Table 5
Pharmacokinetic parameters of methylglyoxal in mice after a single oral dose of methylglyoxal[a,b]

Dose (mg/kg)	Lag time (min)	C^c_{max} (nmol/ml)	t_{max} (h)	k_a (h^{-1})	V (L/kg)	k (h^{-1})	$t_{1/2}$ (h)	CL (L/h.kg)
50	≈55	13.9 ± 2.85	≈4	0.277	23.31	0.33	2.1	7.69
100	≈50	18.7 ± 3.62	≈4	0.223	41.84	0.198	3.5	8.28
200	≈50	19.5 ± 3.36	≈4	0.216	80.97	0.192	3.6	15.54

[a] Values are means.
[b] Two mice were used in each dose level.
[c] Abbreviations: C_{max} – maximum blood concentration, t_{max} – time to C_{max}, k_a– apparent absorption rate constant, V—apparent volume of distribution, CL—apparent total body clearance, k – elimination rate constant, $t_{1/2}$ – elimination half-life.

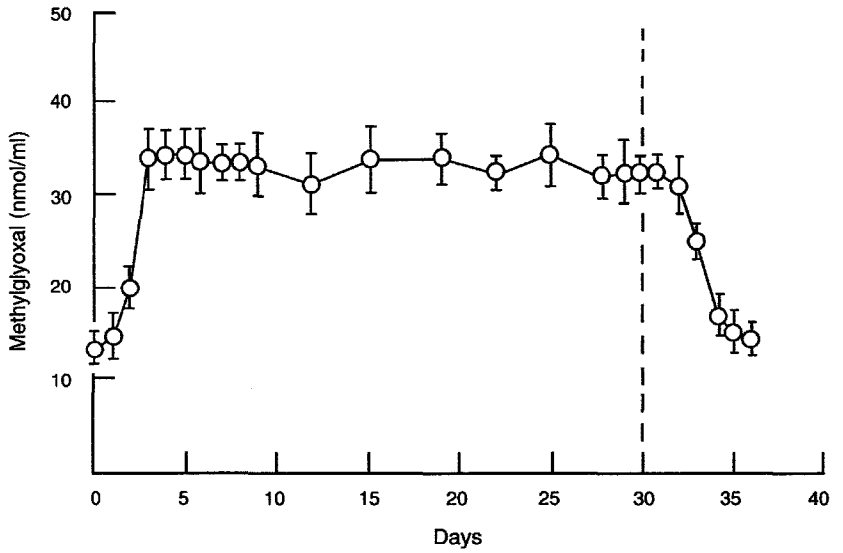

Fig. 3. Blood methylglyoxal concentrations in mouse in repeat oral dose study.

behavioral pattern of all the animals. No death was observed among the animals, and they remained perfectly healthy.

So, we investigated whether, similar to the absence of any apparent external toxic effect, the biochemical functions of some vital organs of the animals remained unchanged. The results for the biochemical studies with rat and dog are presented in Tables 3 and 4 respectively. It appears from Table 3 that treatment of methylglyoxal had no toxic effect on the functions of liver, kidney and heart and hemopoietic organs of the rats. This is indicated by the fact that the values of the respective marker enzymes and metabolites and cells remained unaltered in both control and treated groups of animals.

It is also to be noted that with the exception of alkaline phosphatase and aspartate transaminase the values of different metabolites and marker enzymes measured from the blood/serum of rat were in the range that are usually found in human samples. We checked the serum alkaline phosphatase and aspartate transaminase level in human serum by the same procedure and found that the level of these enzymes in human samples is in the range that is found in normal human serum. These are for alkaline phosphatase and aspartate transaminase 9.4 and 26 units respectively.

In experiments with dog, during and after the treatment (90 days), no death occurred, and the animals appeared perfectly healthy and normal. However, subcutaneous injections appeared to be painful. The biochemical tests and some hematological studies were performed with blood of the animals, the results of which are presented in Table 4.

Pharmacokinetic evaluations

Single dose study

Blood methylglyoxal concentrations after single doses of 50, 100 and 200 mg/kg peaked at 4 h and came to near basal level at 8–10 h (Fig. 2). The basal level of methylglyoxal was determined from the blood of untreated mice and was found out to be around 13.7 ± 2.94 nmol/ml. The individual concentration vs. time curve suggests that absorption and distribution were ongoing processes.

Different pharmacokinetic parameters were determined after a single oral dose of methylglyoxal and are presented in Table 5. It appears from the table that, for all the three doses, 50–55 min after administration of the drug its level began to increase from the basal level (lag time). The maximum concentration of methylglyoxal in blood (C_{max}) after oral administration of methylglyoxal increased from 13.9 to 18.7 nmol/ml when the dose was increased from 50 mg to 100 mg respectively. However, there was a very little increase in C_{max} with a dose of 200 mg as compared to that of 100 mg. The values of C_{max} and other parameters based on this were calculated by subtracting the normal level of methylglyoxal, which was found out to be 13.7 ± 2.94 nmol/ml. The time to reach C_{max} (t_{max}) was found out to be 4 h. Because these results suggest that similar blood concentrations may be observed with daily doses of either 100 or 200 mg, 100 mg was chosen as the dose level for the multiple dose study.

Because the concentration of methylglyoxal in the blood began to rise approximately about 1 h after administration and reached a peak at 4 h, the rate of absorption (k_a) for methylglyoxal was measured from 1 to 4 h after administration of the drug.

Table 6
Pharmacokinetic parameters of methylglyoxal in blood of mice dosed orally by 100 mg/kg body wt./ day of methylglyoxal

Time after last dose (h)	V(L/kg)	k (h^{-1})	$t_{1/2}$ (h)	CL (L/h.kg)
Up to 48	73.90	0.0017	408	0.125
48-120	50.89	0.0231	30	1.175

Abbreviations and symbols are similar to Table 5.

Table 7
Increase in life span of EAC cell_inoculated mice treated with methylglyoxal ascorbic acid and creatine

Treatment group	Median survival time (days)	60 day survivors/15 animals	ILS value (%)
Control	19	Nil	0
MG (100 mg)	Indefinite	10	Cure
MG (50 mg) + AA (50 mg)	Indefinite	11	Cure
MG (30 mg)	26	1	34
MG (30 mg) + AA (50 mg)	32	3	65
MG (30 mg) + AA (50 mg) + Cr (150 mg)	Indefinite	13	Cure
MG (30 mg) + AA (50 mg) + Cr[a] (150 mg)	Indefinite	13	Cure

For this experiment, from a total number of 105 mice, 15 animals received a particular mode of treatment. Control group received only normal saline; the other five groups received methylglyoxal alone or methylglyoxal plus ascorbic acid or methylglyoxal plus ascorbic acid plus creatine. All the test substance was dissolved in 0.9% NaCl and 0.5 ml was separately injected intraperitoneally once day for consecutive 14 days. The day on which 10^5 EAC cells were inoculated into each mouse was considered as day 0. The treatment started from day 3. The amount of each compound indicated in the parentheses is the amount administered per kg body weight per day.
MG – Methylglyoxal; AA – Ascorbic acid, Cr- Creatinine.
[a] Creatine was dissolved in water and the mice were fed instead of injected.

The apparent volume of distribution (V) relates to the amount of drug in the body to the concentration of the drug (C) in the blood. The apparent volume of distribution was calculated from C_p obtained by extrapolation to $t = 0$ ($V = \text{dose}/C_p^0$) considering the body as a single homogeneous compartment, i.e. in one-compartment model.

Clearance or elimination of drug from this compartment occurs in a first order fashion, the amount of drug eliminated per unit time depends on the amount (concentration) of the drug in the body compartment. Then, $C = (\text{dose}/V) \cdot \exp(-kt)$ where k is the rate constant for elimination of the drug from the compartment. This rate constant is inversely related to the half-life of the drug ($k = 0.693/t_{1/2}$).

The semi logarithmic plot (figure not presented) of methylglyoxal concentration in blood versus time appears to indicate that methylglyoxal is eliminated from a single compartment by a first order process with a half life ($t_{1/2}$) of 2.1 to 3.6 h of three different doses (Table 5).

The clearance (CL) for the drug for one compartment model is $CL = k \cdot V$.

The elimination rate constant (k) of methylglyoxal in blood was measured 4–8 h after administration of the drug. This is because the concentration of methylglyoxal in blood reach the maximum (t_{max}) at 4 h after administration and came to the basal level at about 8 h. So the elimination rate constant (k) was determined by measuring methylglyoxal concentration in blood from 4–8 h after its administration.

Similar experiments of single dose study were also done with rats, and the results were similar to the study of the mice (data not presented).

Repeat dose study

In repeat dose study, the peak concentration of methylglyoxal was achieved from day three and remained at that concentration up to day 32 (Fig. 3). The last dose of methylglyoxal was administered on day 30. The peak concentration of methylglyoxal was similar in both single dose and repeat dose study. From day 32, the level of methylglyoxal gradually fell and reached the basal level around day 35.

Different pharmacokinetic parameters are presented in Table 6.

Treatment of cancer-bearing animals

Survival study of EAC cell-inoculated mice treated with methylglyoxal, ascorbic acid and creatine

As mentioned before, methylglyoxal had been found to possess strong antitumor activity. Szent-Györgyi and his associates and Apple and Greenberg long ago showed remarkable antiproliferative and curative effects of methylglyoxal towards cancer-bearing animals (Szent-Györgyi et al., 1967; Együd and Szent-Gtörgyi, 1968; Apple and Greenberg, 1967). In in vivo and in vitro studies with

Table 8
Increase in the percentage of body weight of the EAC cell inoculated mice receiving different treatments

Treatment	Percent increase in body weight				
	Day 15	Day 20	Day 25	Day 30	Day 35
Control (no treatment)	18.3 ± 1.7	36.6 ± 3.9[a]	–	–	–
MG (100)	8.0 ± 0.7	10.0 ± 1.2	11.0 ± 1.0	11.2 ± 0.9	11.2 ± 1.1
MG (50) + AA (50)	7.5 ± 0.7	9.5 ± 0.8	10.4 ± 1.0	10.6 ± 0.8	11.0 ± 1.0
MG (30)	15.8 ± 1.3	19.0 ± 1.6	30.0 ± 2.9	32.9 ± 3.0	38.8 ± 3.0[b]
MG (30) + AA (50)	11.0 ± 1.0	16.4 ± 1.2	18.3 ± 1.3	24.7 ± 1.9	30.2 ± 2.8[c]
MG (30) + AA (50)+ Cr (150)	9.0 ± 0.8	11.0 ± 1.0	11.5 ± 1.0	11.7 ± 1.2	11.9 ± 0.9

For this experiment, from a total number of 36 animals, 6 animals received a particular mode of treatment. The details of the inoculation and treatment schedule were identical to Table 7. The body weight was measured from day 15. The amount of each compound indicated in the table is mg per kg body weight per day.
MG – Methylglyoxal, AA – Ascorbic acid, Cr – Creatinine. a – 2 animals survived out of 6, b – 2 animals survived out of 6, c – 3 animals survived out of 6.

animals and in vitro studies with a wide variety of human post-operative malignant tissue samples, we had observed that methylglyoxal acted specifically against malignant cells and ascorbic acid significantly augmented this anticancer effect of methylglyoxal (Ray et al., 1991, 1997a, 1997b; Biswas et al., 1997). Moreover, we had observed that creatine present in cardiac cells completely protected the animal from any possible deleterious effect of methylglyoxal treatment on cardiac mitochondria (Sinha Roy et al., 2003). So, we tested whether these compounds had any curative effect on mice inoculated with EAC cells. The results are presented in Table 7. It appears that at a particular dose the antiproliferative effect of methylglyoxal is augmented in presence of ascorbic acid and further improved when the mice were treated with methylglyoxal in combination with ascorbic acid and creatine. Nearly 80% of the animals treated with this combination were completely cured.

Measurement of increase in the body weight of the mice inoculated with EAC cells and receiving treatment of methylglyoxal, ascorbic acid and creatine

Measurement of the increase in body weight of the mice inoculated with EAC cells is a good (reliable) indicator of multiplication of EAC cells in the host. Table 8 represents the results of such a study. It appears from the table that similar to the effect of methylglyoxal, methylglyoxal plus ascorbic acid and methylglyoxal plus ascorbic acid plus creatine on median survival time of EAC cell-inoculated mice (Table 7), the maximum arrest of the increase in body weight was observed with treatment of methylglyoxal plus ascorbic acid plus creatine.

Tumor growth inhibition study

We also investigated the relative effect of three different drug combinations on the multiplication of cells in the peritoneal cavity of mice inoculated with EAC cells. Table 9 shows that both methylglyoxal and methylglyoxal plus ascorbic acid treatment had significant inhibitory effect on cell proliferation. Moreover, treatment of methylglyoxal plus ascorbic acid plus creatine not only completely inhibited the cell proliferation but also made the peritoneal cavity completely dry. However, both ascorbic acid and creatine individually at the concentrations mentioned in Table 7 have no effect on EAC cells inoculated mice. Fig. 4 shows the control and treated animals.

Discussion

Here, we have assessed the possible toxic effect of methylglyoxal in in vivo studies with four different species of animals. We administered creatine and/or ascorbic acid along with methylglyoxal in some toxicity studies, and the results were found to be similar where only methylglyoxal was administered. Both ascorbic and creatine are naturally occurring compounds, and their consumption had been found to have no major adverse effect on human (Wyss and Kaddurah-Daouk, 2000). So, we present results where only methylglyoxal was administered. We also present the results of the assessment of the efficacy of methylglyoxal in combination with ascorbic acid and/or creatine to treat cancer-bearing mice.

It appears from the results of acute toxicity studies with mice and rat that methylglyoxal is well tolerated. Three different modes of treatment were used, and the amounts of methylglyoxal administered were many times higher than the intended dose for treatment of cancer patients through the respective routes. The intended dose for the treatment of cancer patients had been worked out from the previous studies of Együd and Szent-Gtörgyi (1968) and of Apple and Greenberg (1967, 1968) and our studies presented in this paper (Tables 7–9). Acute toxicity studies with rat also provided similar results as that of the experiments with mice.

Long-term toxicity studies with four different species of animals had been investigated. For mouse and rabbit, gross physical and behavioral conditions had been noted. Whereas for rat and dog in addition to these observations, several biochemical parameters, which are indicative of the functions of specific organs such as liver, kidney and heart,

Table 9
Tumor growth inhibition study

Treatment	No. of EAC cells (in million)				% of cells present in respect of the control			Volume of packed cells (ml)	
	Day 3	Day 10	Day 14	Day 18	Day 10	Day 14	Day 18	Day 14	Day 18
Control	3.8 ± 0.6	320 ± 20	1800 ± 300	3600 ± 300	100	100	100	1.9 ± 0.3	3.8 ± 0.1
MG (100)	-Do-	d	d	d	0	0	0	n.d	n.d
MG (50) + AA (50)	-Do-	d	d	d	0	0	0	n.d	n.d
MG (30)	-Do-	15 ± 2	230 ± 10	820 ± 30	5	13	23	0.26 ± 0.02	0.96 ± 0.05
MG (30) + AA (50)	-Do-	7.5 ± 1	98 ± 2	360 ± 20	2.4	5.4	10	0.12 ± 0.01	0.4 ± 0.03
MG (30) + AA (50) + Cr (150)	-Do-	d	d	d	0	0	0	n.d.	n.d.

Each group consists 10 mice. Each mouse was inoculated with 10^5 (0.1 million) EAC cells. Treatment with three different drug combinations were started from day 3 and continued up to day 16. The day of inoculation was considered as day 0. The mode of treatment of the drugs was similar to that of Table 7. The ascites fluid containing cells were quantitatively removed from the peritoneal cavity on the indicated day and cell volume and number were counted. The details of collection and counting are described in Materials and Methods. The amount of each drug as indicated in the table is mg per kg body weight per day.
d – Dry; n.d. – Not detectable; MG – Methylglyoxal; AA – Ascorbic acid, Cr- Creatinine.

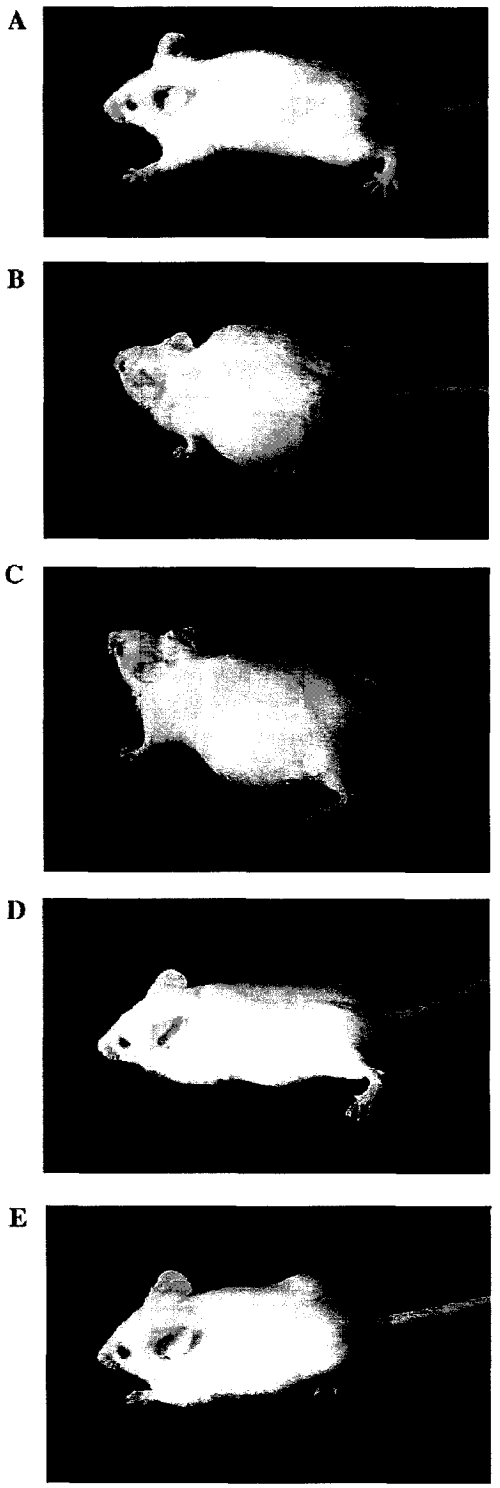

Fig. 4. Effect of methylglyoxal, methylglyoxal plus ascorbic acid and methylglyoxal plus ascorbic acid plus creatine on EAC cell-inoculated mice. Photographs were taken of animals one from each group, EAC cell counts of which are presented in Table 9. Details of inoculation with EAC cells and treatment schedule are described in the legend of Table 9. The photographs were taken on day 18. (A) Normal mouse. (B) Control animal (EAC cell-inoculated, without any treatment). (C) Treated with MG 30 mg. (D) Treated with MG 30 mg + AA 50 mg. (E) Treated with MG 30 mg + AA 50 mg + 150 mg. The drugs applied were /kg body weight/day.

had been assessed. Additionally, histological studies with several organs of mouse, which were orally treated with methylglyoxal, have indicated the respective organs that were apparently devoid of any toxic effect.

It appears from the results presented in Tables 2–4 that, similar to the acute toxicity studies with mouse, the long-term treatment of mouse, rat, rabbit and dog with methylglyoxal had apparently no toxic effect on the gross physical and behavioral conditions of the animals.

The fertility and teratogenicity studies with rat as presented in this paper have also indicated that methylglyoxal has no apparent adverse effects on the reproductive organs of the animals and also on the fetus. In a previous classic experiment, it had also been observed that methylglyoxal-treated mice were indefinite survivors and produced healthy litters (Együd and Szent-Gtörgyi, 1968).

We had also tested the long-term effect of methylglyoxal on several metabolites and marker enzymes in the blood (and serum) of rat and dog (Tables 5 and 6). It had been observed that methylglyoxal treatment had no effect on these metabolites and enzymes. The hemoglobin content of the blood of rat remained unchanged. Methylglyoxal also had no effect on blood glucose and serum urea and creatinine level, indicating no damage of pancreatic cells, and kidney functions were apparently normal. Although it had been reported that in diabetic patients the level of methylglyoxal in blood is higher in comparison to the normal subjects (McLellan et al., 1992), our study indicates that methylglyoxal does not elevate the blood glucose level. Methylglyoxal treatment had also apparently no effect on marker enzymes of hepatic and cardiac functions. The studies with dog and bitch had also yielded similar results (Table 6). It is pertinent to mention here that other investigators had observed methylglyoxal pretreatment through oral route provided dose-dependent protection of gastric mucosa against different necrotizing agents—ethanol, sodium chloride and sodium hydroxide (Al-Shabanah et al., 2000).

In the pharmacokinetic study of methylglyoxal in mice, it had been observed that the compound is appropriately cleared from the system. Moreover, the different parameters that had been worked out from this study can be gainfully utilized in its future human application.

Taken together, these results suggest the possibility of safe administration of methylglyoxal to mammals and administration to cancer patients may also be safe. As mentioned before in the Introduction, numerous reports had recently appeared in the literature, which suggest that methylglyoxal is potentially toxic. It can induce mutation (Murata-Kamiya et al., 2000), advanced glycation end products formation, reactive oxygen species generation (Chan et al., 2005; Chang et al., 2005) and other deleterious effects. However, these are mostly in vitro studies. Our studies presented here and several in vivo studies by other investigators suggest that a thorough study is required to ascertain whether these deleterious effects do indeed occur

in vivo and what are the consequences in intact organisms. In the absence of such study, the present authors feel it will be unwise not to make use of the potentially beneficial effects of methylglyoxal.

As mentioned, the anticancer property of methylglyoxal had been known for a long time. Moreover, Apple and Greenberg (1967, 1968) were able to produce indefinite survivors in mice-bearing tumors on treatment of methylglyoxal at a particular dose level. It had also been reported that ascorbic acid augments the anticancer effect of methylglyoxal (Elvin and Slater, 1981). Ascorbic acid can facilitate the formation of protein aldehyde adduct (Szent-Györgyi, 1979; Tuma et al., 1984). We had also previously observed that, on incubation with methylglyoxal, EAC cells lost their viability and transplantability (Ray et al., 1991). Methylglyoxal also inhibited respiration of a wide variety of malignant cells both human and of animals. Moreover, ascorbic acid significantly augmented this antitumor effect of methylglyoxal (Ray et al., 1991).

There are reports in the literature that creatine and especially its analogue cyclocreatine have anticancer property (Wyss and Kaddurah-Daouk, 2000). We had observed that methylglyoxal inhibited mitochondrial respiration of specifically malignant and cardiac cells but not of other normal cells. Interestingly, methylglyoxal had no effect on the respiration of cardiac tissue slices and several important functions of perfused but intact heart (Ray et al., 1997a, 1997b). These observations suggested that in cardiac cells outside the mitochondria there is a protective device to counteract the possible deleterious effect of methylglyoxal on cardiac mitochondria. Recently, we have identified that creatine is largely responsible for this protective effect (Sinha Roy et al., 2003).

It appears from the survey of literature that the remarkable anticancer effect of methylglyoxal had not been utilized and not even extensively tested. Considering this aspect and also the possible beneficial effect of ascorbic acid and creatine both in augmenting the anticancer effect of methylglyoxal and protecting the host from any adverse effect, we have tested the efficacy of methylglyoxal in combination with ascorbic acid and creatine. The results presented in Tables 7–9 clearly indicate that the anticancer effect of methylglyoxal is significantly augmented in the presence of both ascorbic acid and creatine. A possible explanation for the augmenting effect of ascorbic acid may be provided through its role in the formation of protein aldehyde adduct (Tuma et al., 1984; Szent-Györgyi, 1979). Although novel, we are at present unable to provide any definite explanation for the augmenting effect of creatine. However, there is report in the literature of increased turnover of creatine phosphate in macrophages during phagocytosis, which replenishes the ATP consumed. The creatine provided to the cancer-bearing animals along with methylglyoxal and ascorbic acid may help in augmenting their pool of creatine phosphate in macrophages that is necessary for the phagocytosis of the malignant cells (Loike et al., 1979).

The results presented in this and several other previous publications strongly demand that methylglyoxal alone or in combination with other substances should be tested for the treatment of cancer patients. All the drugs now being used for the treatment of cancer patients are moderate to highly toxic, and their efficacy to arrest growth or to kill malignant cells are often doubtful. The efficacy of a particular drug and also its mode of treatment should be assessed by balancing the benefits and adverse effects. Moreover, without applying to cancer patients mere in vitro experiments and in vivo studies with animals, the efficacy of methylglyoxal cannot be assessed. In fact, a pilot study with methylglyoxal-based formulation on cancer patients had already been done, and the results are promising (Ray et al., 2001). If researchers and clinicians with open mind immediately make a concerted effort to use and to further improve the formulation and treatment, then this dreadful disease from which millions of people are suffering at present throughout the globe may be truly controlled.

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CASE REPORT

Effect of Short-term High-Dose Creatine Supplementation on Measured GFR in a Young Man With a Single Kidney

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It currently is unknown whether creatine supplementation is safe for people with or at risk of kidney disease. We report on the short-term effects of creatine supplementation on kidney function in a young man with a single kidney and mildly decreased glomerular filtration rate (GFR). A 20-year-old man who had undergone unilateral nephrectomy and presented with mildly decreased GFR without kidney damage underwent a trial with 35 days of creatine supplementation (20 g/d for 5 days followed by 5 g/d for the next 30 days) and had his kidney function monitored. After the intervention, ^{51}Cr-EDTA clearance (pre, 81.6 mL/min/1.73 m^2; post, 82.0 mL/min/1.73 m^2), proteinuria (protein excretion: pre, 130 mg/d; post, 120 mg/d), and electrolyte levels were unchanged. Albuminuria, serum urea level, and estimated creatinine clearance were decreased (pre, 4.6 mg/d; post, 2.9 mg/d; pre, 37 mg/d; post, 28 mg/dL; and pre, 88 mL/min/1.73 m^2; post, 71 mL/min/1.73 m^2, respectively), whereas serum creatinine level was slightly increased (pre, 1.03 mg/dL; post, 1.27 mg/dL), falsely suggesting kidney function impairment. This prospective report suggests that short-term creatine supplementation may not affect kidney function in an individual with a single kidney, mild decreased GFR, and ingesting a high-protein diet (ie, 2.8 g/kg/d). This finding has great relevance considering that creatine-induced kidney disease has been a growing concern, even for healthy people.

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INDEX WORDS: Creatine supplementation; kidney disease; adverse effects.

In addition to the well-documented benefits of creatine supplementation on athletic performance,[1] accumulative evidence also suggests that this supplement is capable of attenuating the degenerative state in some muscle, bone, and cartilage disorders; central nervous diseases; and metabolic disturbances.[2] As a result, the use of creatine supplementation has increased worldwide in the last decade.[1,2]

However, the safety of creatine supplementation is controversial, particularly with respect to kidney function. It has been speculated that in individuals receiving creatine, the excessive amount is still a burden to be eliminated mostly by the kidneys because creatine is converted spontaneously to creatinine.[3] Although we and others have repeatedly shown that creatine does not affect kidney function in healthy humans,[4-6] a few case reports have retrospectively observed creatine-induced decreased kidney function, particularly in individuals with preexisting kidney disease.[7,8] In this context, it is uncertain whether creatine might be safe for people with or at risk of kidney disease.

We report on the effects of short-term creatine supplementation on kidney function in a young man with a single kidney and mild decreased glomerular filtration rate (GFR).

CASE REPORT

A 20-year-old man (body weight, 69.3 kg; height, 1.79 m; body fat, 10%; and blood pressure, 120/80 mm Hg) underwent unilateral nephrectomy in 1999 because of a kidney neoplasia. Thereafter, the patient has had kidney function monitored regularly, and mildly decreased GFR has been noticed, as expected for his condition (range of estimated creatinine clearance [eCCr], 71-83 mL/min/1.73 m^2 [1.18-1.38 mL/s/1.73 m^2]). Currently, he has been engaged in intensive resistance training. To enhance the gains in strength and muscle mass, his dietitian initiated a trial with creatine supplementation, and his kidney function was monitored at

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our clinical research center. This study was approved by the local Ethical Committee (University of Sao Paulo, Brazil), and written informed consent was given by the patient.

The patient received 20 g/d of creatine monohydrate supplementation for 5 days divided into 4 equal doses, followed by single doses of 5 g/d for the next 30 days. The patient was questioned weekly to determine adherence to the creatine supplementation dosing schedule. To verify the purity of the creatine used, a sample was analyzed using high-performance liquid chromatography.[9] This established 99.9% purity, with no other peaks detected.

At baseline and after 35 days, blood samples were obtained from an antecubital vein after a 12-hour overnight fast, and 24-hour urine samples were collected. We assessed serum creatinine and serum urea using commercial enzymatic colorimetric tests (Labtest, www.labtest.com.br), urinary and serum sodium and potassium using a flame photometer (model 143; Instrumentation Laboratory, www.ilus.com), 24-hour albuminuria and proteinuria using an immunoturbidimetric test, and protein intake using three 24-hour dietary recalls undertaken on separate days (2 weekdays and 1 weekend day). eCCr was calculated using the Cockcroft-Gault formula. Additionally, we performed ^{51}Cr-EDTA clearance measurements. After a protein-restricted diet and 12-hour overnight fast, the patient was admitted to our clinical research center. He rested supine with an indwelling polyethylene catheter inserted into a cubital vein in both arms. A single dose of 3.7 MBq (100 μCi) of the ^{51}Cr-EDTA tracer in a volume of 1 mL was injected intravenously into the right arm. The catheter was flushed with 10 mL of saline. Accurately timed 10-mL blood samples were drawn into a heparinized tube from the opposite arm at 4 and 6 hours after the injection. The plasma disappearance curve was constructed using results of these times. To measure radioisotope activity, blood samples were centrifuged at 1,500*g* for 10 minutes, and 3 mL of plasma measured in a well-counter calibrated for the energy of chromium 51 (320 keV). Each sample, including a 3-mL standard obtained as an aliquot from 3.7 MBq (100 μCi) of ^{51}Cr-EDTA diluted to 500 mL in saline, was counted for 5 minutes. Plasma clearance rate was calculated using the slope-intercept method with a single-compartment model, which assumes that the tracer spreads out immediately after injection in its volume of distribution. The Brochner-Mortensen method was used for correcting the systematic error of the slope-intercept technique according to the following equation:

$$Cl_c = 0.9908 \times Cl_{nc} - 0.001218 \times Cl_{nc}^2$$

where Cl_c is clearance corrected for the first exponential and Cl_{nc} is the noncorrected clearance. ^{51}Cr-EDTA clearance and eCCr also were corrected for body surface area.

All samples were analyzed in duplicate, and coefficients of variation were 2%, 2.2%, 1.1%, 2.1%, 2.3%, 5.3%, 9.7%, 24.5%, and 16.4% for creatinine, serum sodium, serum potassium, serum urea, proteinuria, albuminuria, ^{51}Cr-EDTA clearance, urinary sodium, and urinary potassium, respectively.

At baseline, the patient presented with mildly decreased GFR (^{51}Cr-EDTA, 81.6 mL/min/1.73 m^2 [1.36 mL/s/1.73 m^2]) without evidence of kidney damage. After 35 days of creatine supplementation, ^{51}Cr-EDTA clearance, electrolyte levels, and proteinuria were virtually the same (ie, variation less than the coefficient of variation). Albuminuria, serum urea level, and eCCr decreased 37%, 25%, and 19.3%, respectively. Serum creatinine level increased 23.3% (Table 1). Protein intake was 2.7 g/kg/d at baseline and 2.8 g/kg/d after creatine supplementation. The patient reported that adherence to the creatine supplementation protocol was 100%, which is consistent with the rapid weight gain seen after the trial (2.5 kg).

Table 1. Effects of 35-Day Creatine Supplementation on Kidney Function in a 20-Year-Old Man With a Single Kidney and Mildly Decreased Glomerular Filtration Rate

	Before Creatine Supplementation	After Creatine Supplementation
^{51}Cr-EDTA clearance (mL/min/1.73 m^2)	81.6	82.0
Serum creatinine (mg/dL)	1.03	1.27
eCCr (mL/min/1.73 m^2)	88	71
Albuminuria (mg/d)	4.6	2.9
Proteinuria (mg/d)	130	120
Serum urea (mg/dL)	37	28
Serum sodium (mEq/L)	142	143
Serum potassium (mEq/L)	4.6	4.2
24-h Urinary sodium (mEq/L)	228	289
24-h Urinary potassium (mEq/L)	61	70

Note: Conversion factors for units: serum creatinine in mg/dL to μmol/L, ×88.4; serum urea in mg/dL to mmol/L, ×0.166; CCr in mL/min/1.73 m^2 to mL/s/1.73 m^2, ×0.01667, no conversion necessary for serum sodium and potassium levels expressed in mEq/L and mmol/L.

Abbreviation: eCCr, estimated creatinine clearance calculated using the Cockcroft-Gault equation.

DISCUSSION

We show for the first time that short-term creatine supplementation may be safe in an individual with a single kidney and mildly decreased GFR. This finding has great relevance considering that creatine-induced kidney disease has been a growing concern, even for healthy people (ie, without decreased kidney function).

A few case reports suggested that creatine supplementation could cause deterioration in kidney function in individuals with[7] or without[10] preexisting kidney disease. Nonetheless, these studies have severe limitations, such as lack of gold-standard measures for assessing GFR and a retrospective design. However, longitudinal stud-

ies have shown no deleterious effects of creatine supplementation,[4-6] although they also have been criticized for limited statistical power and low sensitivity of the methods used to evaluate GFR. Importantly, no human study has examined prospectively the effects of creatine intake on kidney function in people at risk of kidney disease. This prospective report suggests that short-term creatine supplementation may not affect kidney function, even in an individual who has undergone nephrectomy, has mildly decreased GFR, and is ingesting a high-protein diet (ie, >3 times more than the recommended intake for healthy people). This finding is consistent with the observation that creatine intake as high as 5-7 g/d in some diets[11] has no apparent effect on kidney function. It would be surprising if, given the span of human evolution, some adaptation to creatine intake had not occurred. The rapid and complete absorption of creatine supplied in the diet,[11,12] in contrast to the lack of absorption in a herbivorous animal, such as the horse,[13] suggests that adaptation has occurred at least at one level.

It is interesting to note that serum creatinine level seemed to be slightly increased after creatine supplementation. Serum creatinine level has become the most commonly used marker of kidney function. However, because creatine is converted spontaneously to creatinine,[3] use of creatine supplements to increase tissue creatine levels invariably will increase creatinine production, falsely suggesting kidney function impairment. Confirming this assumption, we previously observed increased serum creatinine, but normal cystatin C levels after 3 months of creatine supplementation in healthy male individuals.[4] In view of this, we emphasize that use of serum creatinine level to estimate GFR may be inadequate in people supplemented with creatine.

This case study has some limitations. Caution should be exercised in extrapolating these findings to other people with or at risk of kidney disease (eg, elderly patients or those with diabetes). Additionally, larger doses and longer periods of creatine intake may produce different results. Randomized controlled trials using accurate measures for GFR evaluation are necessary to better elucidate the effects of creatine on kidney function, especially in people with or prone to kidney disease. Meanwhile, systematic assessment of kidney function after long-term creatine supplementation is still crucial.

In conclusion, we show that short-term creatine supplementation seems to not affect kidney function in a young man with a single kidney, mildly decreased GFR, and ingesting a high-protein diet.

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Differential effect of creatine on oxidatively-injured mitochondrial and nuclear DNA

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Abstract

Creatine is a naturally occurring compound obtained in humans from endogenous production and consumption through the diet. It is used as an ergogenic aid to improve exercise performance and increase fat-free mass. Lately, creatine's positive therapeutic benefits in various oxidative stress-associated diseases have been reported in literature and, more recently, creatine has also been shown to exert direct antioxidant effects. Oxidatively-challenged DNA was analysed to show possible protective effects of creatine. Acellular and cellular studies were carried out. Acellular assays, performed using molecular approaches, showed that creatine protects circular and linear DNA from oxidative attacks. Nuclear and mitochondrial DNAs from oxidatively-injured human umbilical vein endothelial cells were analyzed. Creatine supplementation showed significant genoprotective activity on mitochondrial DNA. This evidence suggests that creatine may play an important role in mitochondrial genome stability in that it could normalize mitochondrial mutagenesis and its functional consequences. Thus, creatine supplementation could be used to prevent or ameliorate diseases related to mitochondrial DNA mutations, and possibly to delay aging.

Keywords: Creatine; Oxidative DNA damage; Mitochondrial DNA; Nuclear DNA; Antioxidant

1. Introduction

Creatine (Cr) is the most popular supplement proposed as an ergogenic aid. It is distributed throughout the body with 95% found in skeletal muscle and the remaining 5% in the brain, liver, kidney, and testes [1]. Cr is obtained through diet (~1 g/day for an omnivorous diet) and synthesized in the liver, kidney and pancreas (~1 g/day). The dietary intake and endogenous production of Cr match the spontaneous degradation of phosphoCr and Cr to creatinine at a rate of 2.6% and 1.1% per day, respectively. Once creatinine is formed, it enters the circulatory system by diffusion and is eliminated from the body through glomerular filtration. Intramuscular and cerebral stores of Cr, as well as its phosphorylated form, phosphoCr, increase with oral Cr-supplementation. The increase of these stores can offer therapeutic benefits by preventing ATP depletion, stimulating protein synthesis or reducing protein degradation, and stabilizing biological membrane [2].

Evidence from exercise literature has shown that athletes benefit from supplementation by increasing muscular force and power, reducing fatigue in repeated bout activities, and increasing muscle mass [3–7]. In the 1990s Cr supplementation became a popular ergogenic aid for many athletes to maintain a rapid rate of adenosine triphosphate (ATP) turnover during a brief period of high intensity activity [8–15].

In a different direction, Lawler et al. reported that Cr is capable of directly quenching aqueous radical and reactive

Abbreviations: CCC, Covalently closed circular; Cr, Creatine; DTT, DL-dithio-threitol; MFO, Mixed-function oxidase; mtDNA, Mitochondrial DNA; nDNA, Nuclear DNA; 8-OHdG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; PBS, Phosphate-buffered saline; ROS, Reactive oxygen species; PCR, Polymerase chain reaction; QPCR, Quantitative polymerase chain reaction

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species ions *in vitro* [16]. A more recent study from our laboratory showed that Cr exerts direct antioxidant activity *via* a scavenging mechanism in oxidatively-injured cultured mammalian cells [17]. Moreover, other authors have demonstrated that Cr supplementation not only improves exercise performance and increases fat-free mass, but is also beneficial in the prevention or treatment of some oxidative stress associated diseases [18–21]. In these diseases mitochondrial DNA (mtDNA) represents an important target for oxidative damage. Indeed, mtDNA mutations have recently been reported as being an etiological factor in oxidative stress-related disorders [22] including cardiovascular diseases and inherited [23] or acquired [24–26] neurodegenerative disorders, several types of tumors affecting the colon, bladder, lung, breast, kidney, head and neck [27–34] mitochondrial myopathies [35] and the normal aging process [36].

The human mitochondrial genome, completely sequenced in 1981 [37], is a 16,569-bp closed circular, duplex molecule present in a high copy number per cell, widely varying among cell types. It encodes 13 polypeptides, 22 transfer RNAs and 2 ribosomal RNAs, all essential for electron transport and ATP generation [38]. mtDNA has been observed to be more susceptible to damage than nuclear DNA because of several possible factors such as exposure to high levels of reactive oxygen species (ROS) produced during oxidative phosphorylation [39], lack of protective histones, and limited DNA repair pathways [40] having a robust base excision repair (BER) system but not nucleotide excision repair (NER) [41]. Oxidative damage to mtDNA may lead to loss of membrane potential, reduced ATP synthesis and cell death [42]. Mitochondria, being mediators of cell life and death, should therefore represent a potential target for new therapeutic approaches. Indeed, strategies are being developed for the targeted delivery of antioxidants or other cytoprotective agents to mitochondria. Cr might be a possible candidate as a mitochondrially-targeted antioxidant, in that it is actively taken up by mitochondria through specific transporters [43], unlike conventional antioxidants which have limited efficacy due to the difficulty of accumulating within these organelles. However, it is not known whether Cr protects from functionally relevant oxidative-induced mutations of mtDNA.

The aim of the present study is to evaluate possible protective effects of Cr on oxidatively-injured nuclear DNA (nDNA) and mtDNA in an attempt to better understand Cr physiology and to envisage its possible use in the prevention or amelioration of a wide range of oxidative stress-related human diseases where oxidative mtDNA damage plays an etiological role.

2. Materials and methods

2.1. DNA and chemicals

The plasmid pGEM-T (3000 bp) was purchased from Promega. All reagent grade chemicals were obtained from Sigma-Aldrich Inc. All primers were obtained from Sigma-Genosys Inc. and DL-dithio-threitol (DTT) from Clontech. H_2O_2, Fe^{2+}, Fe^{3+} and Cr were freshly prepared for each experiment.

2.2. Treatment of DNA with hydroxyl radicals and Cr

Different DNA sources (pGEM-T, PCR product, genomic DNA) were mixed with freshly prepared DTT (10 mM) and $FeCl_3$ (3 μM) (thiol/Fe^{3+}/O_2 mixed-function oxidase, MFO system) or H_2O_2 (2 mM) and Fe_2SO_4 (3 μM) (Fe^{2+}/hydrogen peroxide, H_2O_2 system) in the presence or absence of different Cr concentrations (from 1 mM to 10 mM), in a total volume of 20 μl of 40 mM HEPES (pH 7). Reaction mixtures were incubated for 100 min at 37 °C. DNA samples were applied to 0.8% agarose/Tris Borate EDTA (TBE) gel, stained with

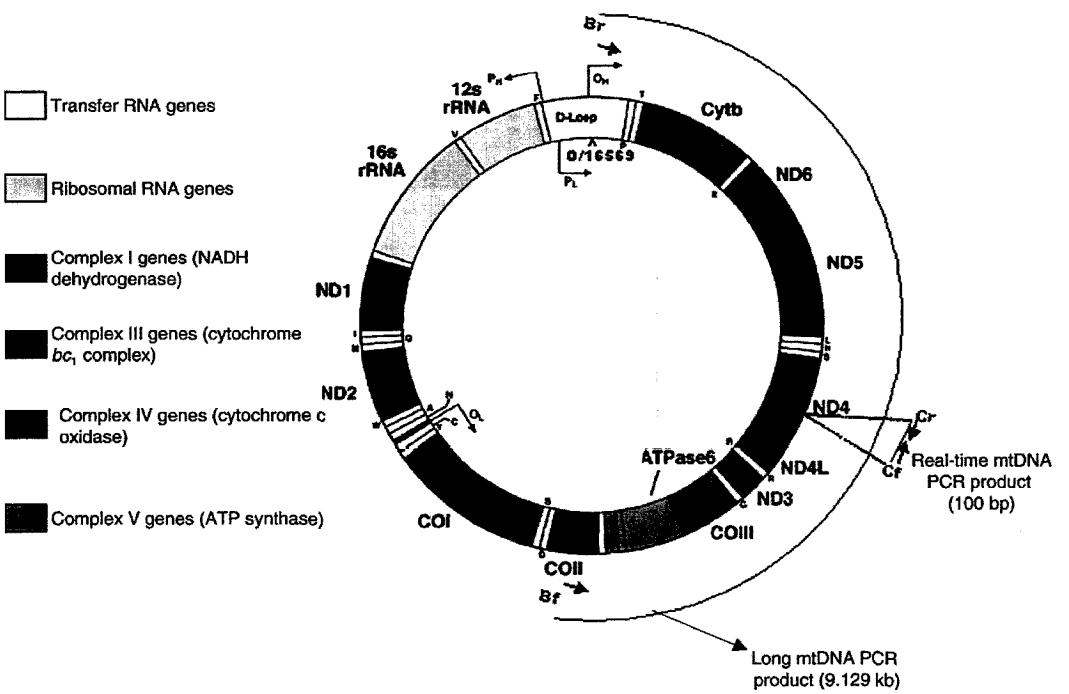

Fig. 1. Schematic representation of human mitochondrial genome and regions amplified by PCR. The four primers used for QPCR and Real-time PCR are indicated by arrows.

ethidium bromide (0.3 μg/ml) and visualized under UV light. Quantification was made by densitometric analysis using Quantity One Software 4.01 (Bio-Rad).

2.3. Isolation of total DNA

High molecular weight DNA was isolated with the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. Total cellular DNA concentration was determined at 260 nm using spectrophotometer (Beckman DU- 640) and nanodrop spectrophotometer (ND-1000 Nanodrop Technologies).

2.4. Cell culture and treatment conditions

HUVEC (human umbilical vein endothelial cells) were cultured at 37 °C in an atmosphere of 95% air and 5% CO_2 in M199 medium containing antibiotics, 1.4 mM glutamine, 10% fetal bovine serum and 50 μg/ml endothelial cell growth factor. HUVEC were seeded at an appropriate density 30–36 h before treatments. At the oxidative challenge stage, the cell number was between 3.5 and 4.5×10^5 cells/well. Cr (10 mM), Trolox (100 μM) or *o*-phenanthroline (10 μM) were added to complete culture medium and were given to cells 24 h, 10′ and 1 h prior to the challenge with H_2O_2, respectively. Trolox and *o*-phenanthroline were also added at the same concentrations to Saline A during treatment with H_2O_2. Oxidative challenge consisted in 30 min incubation of antioxidant-free or antioxidant-supplemented cells with 200 μM H_2O_2 at 37 °C in 2 ml of Saline A (0.145 M NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 5 mM glucose). After treatments, cells were washed with phosphate-buffered saline (PBS, 8 g/l NaCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 0.2 g/l KCl), harvested by trypsination and processed for DNA damage or recultured in the original medium at different times for DNA repair and cytotoxicity studies.

2.5. Trypan blue exclusion assay

Monolayers were detached by trypsinization, an aliquot of cell suspension was diluted 1:1 with 0.4% trypan blue and cells were counted with a hemocytometer. Results are expressed as the number of viable (unstained) cells in treated and control samples.

2.6. Long PCR

Long PCR was performed in a final volume of 25 μl using a Mycycler machine (Bio-Rad). Specific primers were used to amplify a 9.129-kb fragment of the mitochondrial DNA (primers Bf–Br, long mtDNA PCR, Fig. 1) and a 7.3-kb fragment of the nuclear gene GBA (glucosidase, beta acid; primers Df–Dr, long nDNA PCR).

The reaction mixture contained 60 ng template total DNA, 2.5 μl buffer 1, 200 μM dNTPs, 0.5 μM of each primer and 1.5 U of Expand Long Template PCR system (Roche). The primer nucleotidic sequences and PCR parameters are reported in Tables 1 and 2.

Table 1
Primers employed

604 bp from D-Loop mitochondrial DNA (J01415):	
Af 35	5′ GGAGCTCTCCATGCATTTGG 3′
Ar 620	5′ GGGTGATGTGAGCCCGTCTA 3′
9.129 kb mitochondrial fragment (J01415):	
Bf 8080	5′ CCCCACATTAGGCTTAAAAACAGAT 3′
Br 620	5′ GGGTGATGTGAGCCCGTCTA 3′
100 bp mitochondrial fragment (J01415):	
Cf	5′CCATTCTCCTCCTATCCCTCAAC 3′
Cr	5′ CACAATCTGATGTTTTGGTTAAACTATATTT 3′
7.3 kb nuclear DNA of the GBA gene (NM_001005741):	
Df	5′ TTCTCCATGCAAATCTGTGT 3′
Dr	5′ GAACCAGATCCTATCTGTGC 3′
100 bp nuclear DNA of the GBA gene (NM_001005741):	
Ef	5′ AGCATCAGGGCGGAAGC 3′
Er	5′ TTTCTCCTTTAAGAGCTGCCATTT 3′

Table 2
Thermal cycling parameters

	Cycles	Temperature (°C)	Time
PCR 1	1×	95	5 min
		95	30 s
	30×	62	30 s
		72	45 s
	1×	72	7 min
	1×	4	hold
PCR 2	1×	95	2 min
	30×	95	30 s
		68	9 min
	1×	68	12 min
	1×	4	hold
PCR 3	1×	93	2 min
	10×	93	10 s
		60	30 s
		68	4 min 40 s
	20×	93	10 s
		60	30 s
		68	4 min 40 s + 20 s per cycle
	1×	68	7 min
	1×	4	hold
PCR 4	1×	95	10 min
	40×	95	1 min
		60	9 s
	1×	4	hold

2.7. Quantitative PCR (QPCR)

The amplification products obtained by long PCR were electrophoresed on 0.8% agarose/TBE gel, stained with ethidium bromide (0.3 μg/ml) and quantified by densitometric analyses of the intensity of bands using Quantity One Software 4.01 (Bio-Rad). Treated samples were then compared with controls and the relative amplification was calculated according to Santos et al. [44].

Results presented herein are the mean of two sets of PCR for each target of at least three different biological experiments.

2.8. PCR product purification

The Af–Ar mtDNA amplification products (Tables 1 and 2) were purified using the GenElute PCR Clean-up kit (Sigma-Aldrich Inc) according to the manufacturer's instructions.

2.9. Quantitative Real-Time PCR

Long mtDNA and nDNA PCR products, obtained from HUVEC DNA, were also quantified by Sybr Green Real-Time PCR, using primers Cf-Cr and Ef-Er, respectively, localized in the middle of the long PCR fragments (Table 1). After long amplification, samples were diluted 10^{-4}, while the corresponding genomic DNA samples were diluted 10^{-3}.

Quantitative Real-Time PCR was performed in a Bio-Rad iCycler iQ Multi-Color Real-Time PCR Detection System using 2× Quantitect SYBR Green PCR kit (Qiagen). The quantitative PCR reaction was performed at 95 °C for 10 min to activate HotStart DNA polymerase followed by 50 cycles of the two-step at 95 °C for 30 s and at 60 °C for 30 s. The specificity of the amplification products obtained was confirmed by examining thermal denaturation plots and by sample separation in a 3% DNA agarose gel.

Results were normalized by quantitating each sample for the amount of initial genomic DNA without previous long PCR amplification, in the same real-time PCR conditions.

Each sample was tested in triplicate, and the experimental groups (control DNA, H_2O_2-treated DNA with and without Cr time 0, after 2, 4, 24 and 48 h recovery) consisted of at least three independent experiments. The significance

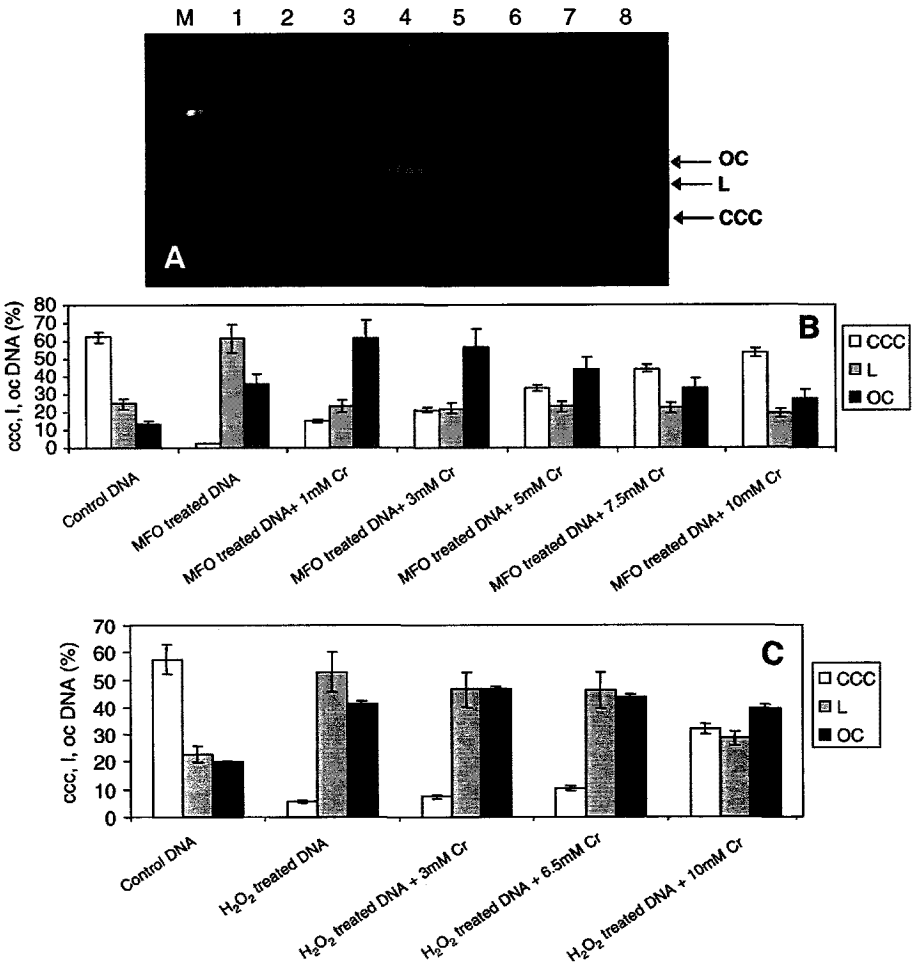

Fig. 2. Protective effect of Cr at different concentrations on plasmid DNA strand breaks induced by the MFO (A, B) and H_2O_2 systems (C). (A) Example of electrophoretic pattern obtained from MFO treatment. Lane M: DNA molecular weight λ/*Hin*dIII; lane 1: control DNA; lane 2: 100 min MFO-treated DNA; lane 3: 0 min MFO-treated DNA+5 mM Cr; lane 4: 100 min MFO-treated DNA+5 mM Cr; lane 5: 0 min MFO-treated DNA+7.5 mM Cr; lane 6: 100 min MFO-treated DNA+7.5 mM Cr; lane 7: 0 min MFO-treated DNA+10 mM Cr; lane 8: 100 min MFO-treated DNA+10 mM Cr. (B, C) Quantitation of Cr protection against DNA strand breaks induced by MFO (B) and Fe^{2+}/hydrogen peroxide (C) systems. Results are expressed as the percentage of the three forms on total plasmid DNA (CCC+OC+L). Values are means±S.E.M. of results from three replicates.

of the difference in undamaged products among the groups has been evaluated by the Wilcoxon signed-rank test.

2.10. Determination of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OHdG) by ELISA assay

DNA was extracted from HUVEC using the DNA Extractor WB Kit (Wako, Osaka, Japan) and suspended in 135 μl of 20 mM of sodium acetate (pH 4.8), digested to nucleotide with nuclease P1 (40 U/ml) at 37 °C for 1 h. Then, 15 μl of 1 M Tris–HCl (pH 7.4) was added to the samples and they were treated with alkaline phosphatase (25 U/ml) at 37 °C for 1 h. 8-OHdG levels in digested DNA were determined using 8-OHdG ELISA kit (Japan Institute for Control Aging, Japan), provided by LiStarFISH S.r.l. (Milano, Italy) [45], according to the manufacturer's instructions. The hydrolysates were filtered through Millipore Microcon YM-10 at 14.000 rpm for 10 min to remove enzymes and other macromolecules. The absorbance values for the test samples were read at 450 nm using the microplate reader Model 680 (Biorad) and the Microplate Manager Software Version 5.2.1 (Biorad).

2.11. Fast halo assay

The assay has been carried out as previously described [46]. Briefly, after the treatments, the cells were resuspended at 4.0×10^4/μl in ice-cold PBS containing 5 mM EDTA: this cell suspension was diluted with an equal volume of 2% low-melting agarose in PBS and immediately sandwiched between an agarose-coated slide and a coverslip. After complete gelling on ice, the coverslips were removed and the slides were immersed in NaOH 300 mM for 15 min at room temperature. Ethidium bromide (10 μg/ml) was directly added to NaOH during the last 5 min of incubation. The slides were then washed and destained for 5 min in distilled water. The ethidium bromide-labelled DNA was visualized using a Leica DMLB/DFC300F fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and the resulting images were digitally recorded on a PC and processed with an image analysis software (Scion Image). The amount of fragmented DNA diffusing out of the nuclear cage, i.e. the extent of strand scission, was quantified by calculating the nuclear diffusion factor, which represents the ratio between the total area of the halo and nucleus and that of the nucleus. Data are expressed as relative nuclear diffusion factor, calculated by subtracting the nuclear diffusion factor of control cells from those of treated cells.

3. Results

3.1. Acellular experiments

We investigated the effects of Cr in acellular systems using different DNA sources, such as plasmid DNA and amplification

products obtained by polymerase chain reaction, which represent circular and linear DNAs, respectively. Fe^{2+}/hydrogen peroxide and thiol/Fe^{3+}/O_2 mixed-function oxidase were used as oxidative systems. In this latter system, autoxidation of thiols (DTT) in the presence of iron generates reactive oxygen species (ROS) such as superoxide anion, H_2O_2 and hydroxyl radical ($OH^{\cdot}$) [47]. In particular, hydroxyl radical generated in close proximity to nucleic acid molecules can add hydrogen atoms to DNA bases or abstract hydrogen atoms from the sugar moiety leading to modified bases, DNA strand breaks or abasic sites [48].

Aliquots of 600 ng pGEM-T plasmid were used in order to investigate and assess the induction of single and double strand breaks in the covalently closed circular (CCC) form. In DNA preparations from prokaryotic cells the plasmids show three topoforms, being the prevalent CCC, which may be converted in relaxed (open circular) or linear forms due to single or double strand breaks during experimental manipulations (Fig. 2). Control DNA, H_2O_2 and MFO treated samples in the absence or presence of Cr were assayed through 0.8% agarose gel electrophoresis to quantify the three plasmid topoforms. Electrophoresis analysis showed that both oxidation systems perturbed plasmid stability with a drastic reduction of the supercoiled form and an increase of circular and linear forms, indicating the development of single and double strand breaks, respectively. The addition of Cr resulted in a partial inhibition of the conversion of supercoiled to linear and open circular forms in a dose-dependent manner (Fig. 2).

In another in vitro assay a 600-bp region amplified by polymerase chain reaction (primers Af-Ar in Table 1 and PCR1 in Table 2) was treated as described for the plasmid DNA. The amplification product treated for 100 min with oxidant systems with or without Cr did not show any difference in the agarose-gel electrophoresis patterns. Nevertheless, after an overnight exposure Cr-free samples showed an almost complete DNA degradation while Cr-supplemented samples were partially protected (Fig. 3).

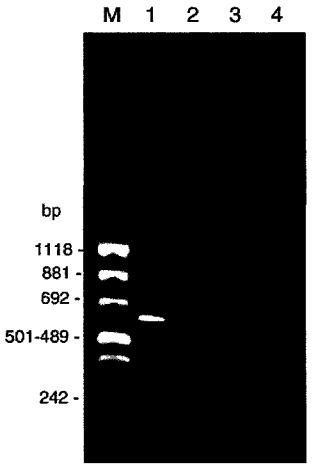

Fig. 3. Protective effect of Cr on a MFO-treated 600 bp mtDNA amplification product. Lane M: DNA molecular weight pUC Mix Marker 8; lane 1: control amplified product; lane 2: no template control; lane 3: MFO-treated PCR product; lane 4: MFO-treated PCR product in the presence of 10 mM Cr.

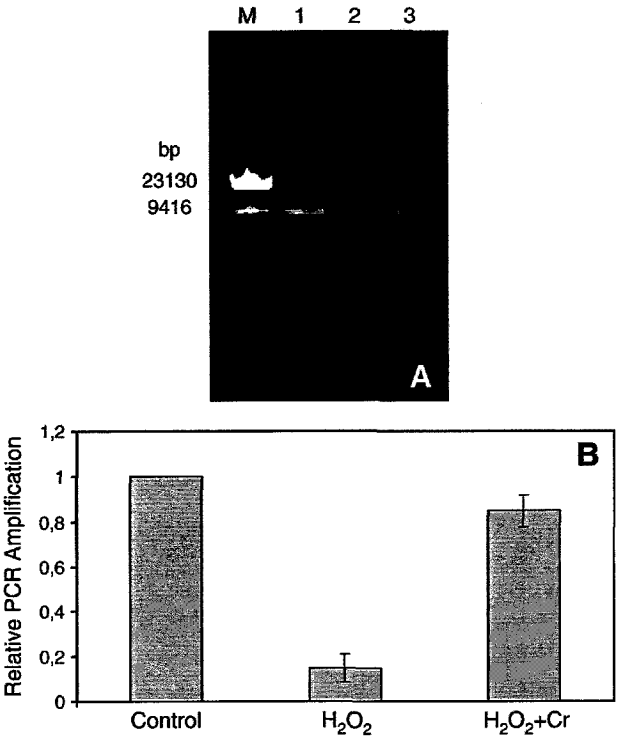

Fig. 4. Protective effect of Cr on mtDNA. (A) Electrophoretic profile of a 9-kb mtDNA region PCR. Lane M: DNA molecular weight λ/*Hin*dIII; lane 1: control amplified product; lane 2: H_2O_2-treated PCR product; lane 3: H_2O_2-treated PCR product in the presence of 10 mM Cr. (B) Quantitation by densitometric analysis of the PCR products obtained in panel A. Values are means±S.E.M. from at least three independent experiments.

The results obtained on these different DNA sources show that DNA damage develops at different times suggesting that both damage and Cr protection could be related to the conformation of the DNA source under analysis.

In a further acellular experiment, 60 ng of total genomic DNA were treated with Fe^{2+}/H_2O_2 in the absence or presence of Cr and then used as a template for the amplification of a long mtDNA region with a proofreading DNA polymerase (Table 2, PCR 2) to evaluate the extent of DNA damage. This technique is based on the premise that DNA lesions, including oxidative damage such as strand breaks, abasic sites and some base modifications (for example, 8oxodA) can hamper the progression of the polymerase [49,50]. The reduction in the relative yield of the long PCR product reflects the presence of blocking DNA lesions [44,51] rather than the exhaustion of a critical reagent. Thus, amplification is inversely proportional to DNA damage: the more lesions on the DNA target, the less amplification. The amplification products obtained by long PCR were quantified by densitometric analysis, after gel electrophoresis migration, obtaining a quantitative PCR (QPCR). To obtain a real quantitative assay it is necessary to detect the cycle number at which amplification products are in the exponential phase of the long PCR: this condition ensures that other components of the reaction such as dNTPs, primers and Taq polymerase are not limiting. Hence, the cycle number selected assures that by amplifying a 50% control (containing half of the amount of the

non-damaged template, that is 30 ng), a ~50% reduction of the amplification signal is obtained, as compared to a 100% control (data not shown). The protective effect of Cr was also observed in this assay (Fig. 4A, B).

3.2. Cell studies

The genoprotective effect of Cr in DNA acellular systems led us to investigate whether similar effects could be observed in nDNA and mtDNA from H_2O_2-injured cells.

HUVEC cells, which had been previously shown to be sensitive to the antioxidant activity of Cr [17] were preincubated for 24 h with or without 10 mM Cr and then treated for 30 min with 200 μM H_2O_2. The number of viable cells was determined after 24, 48 and 72 h of growth in fresh culture medium using the trypan blue exclusion assay. Under these conditions Cr-supplementation afforded significant and durable cytoprotection in H_2O_2-treated cells (Fig. 5).

In order to quantify the formation of nDNA and mtDNA lesions, the efficiency of mtDNA damage removal as well as the effect of Cr-supplementation, DNA analyses were performed. DNA from *o*-phenanthroline or trolox supplemented cells exposed to H_2O_2 was also analyzed being *o*-phenanthroline a reference iron chelator and Trolox a reference radical scavenger.

The QPCR and the Real-Time PCR were the strategies selected to quantify the yield of the long mtDNA PCR products and nDNA PCR products from H_2O_2-treated, Cr-supplemented or unsupplemented HUVEC cells. One of the main advantages of the QPCR and Real-Time PCR assays is that they allow monitoring of the integrity of mtDNA directly from total cellular DNA avoiding processes which can increase base oxidation such as isolation of mitochondria or separate mtDNA purification steps.

QPCR showed that oxidative challenge induces more extensive damage in mtDNA (Primers B in Table 1 and PCR 2 in Table 2) than in nDNA (Primers D in Table 1 and PCR 3 in Table 2) and that Cr protective effect is statistically significant only on mtDNA (Fig. 6).

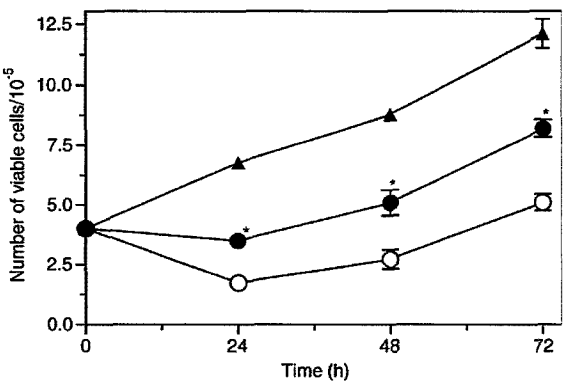

Fig. 5. Effect of Cr preloading on oxidant-induced cytotoxicity in HUVEC cells. Cells were pre-incubated for 24 h in the absence (open circles) or presence (closed circles) of 10 mM Cr, and then treated for 30 min in Saline A with 200 μM H_2O_2. Also shown is the growth curve of control cells (triangles). The number of viable cells was determined after 24, 48 and 72 h of growth in fresh culture medium using the Trypan blue exclusion assay (see Materials and methods). Results represent the means±S.E.M. from at least 5 separate experiments. * $P<0.005$ (unpaired *t* test) compared to Cr unsupplemented cells.

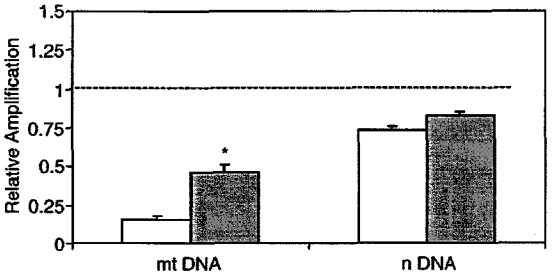

Fig. 6. QPCR on mt and nDNAs from Cr-supplemented (10 mM) and unsupplemented HUVEC. Cells were pre-incubated for 24 h in the absence (open bars) or presence (solid bars) of 10 mM Cr, treated for 30 min in Saline A with 200 μM H_2O_2 and immediately assayed for QPCR. The decrease in amplification was calculated comparing treated samples to undamaged control (dashed line). Data are expressed as the mean±S.E. of at least three separate determinations in which two Long PCRs were performed per experiment. Student's *t* test was performed comparing controls to H_2O_2-treated groups or Cr untreated to treated groups. $P<0.05$ when controls and H_2O_2-treated groups were compared. *, $P<0.05$ comparing mtDNA from H_2O_2-treated cells to mtDNA from 10 mM Cr supplemented H_2O_2-treated cells.

Primer pairs located in the middle of mitochondrial and nuclear long PCR fragments were used in the Real-Time PCRs (Primers C and E in Table 1 and PCR 4 in Table 2). Standard curves have been established between DNA quantities used as templates in the long PCR and PCR amplicons detected by Real-Time PCR. Results were normalized by quantifying each sample for the amount of initial DNA without previous long PCR amplifications. This two-step assay was selected because a standard Real-Time PCR which uses total DNA, without previous long PCR amplification, produces overlapping curves either with or without induced DNA damages, since the amplification product is too short to show DNA polymerase proofreading blocking. These results were similar to those obtained with QPCR. Indeed, only with mitochondrial primers, significant shifts of the Real-Time PCR amplification curves to the right were obtained in H_2O_2-treated samples when they were compared with controls, reflecting the decrease in the long PCR and the presence of DNA lesions (Fig. 7A); notably, shifts to controls were obtained in Cr supplemented samples reflecting Cr protection and minor DNA damage on mitochondrial DNA with respect to H_2O_2-treated samples (Fig. 7A). Shifts to controls were not significant with nuclear primers suggesting that Cr is not protective on nuclear DNA.

The effect of Cr on mtDNA, as assayed immediately after oxidative challenge, was dose-dependent in the 3- to 10-mM range; concentrations lower than 3 mM were ineffective (not shown). Moreover, the protection afforded by 10 mM Cr, the dose used throughout this set of experiments by virtue of its higher activity, was significant from 0 up to 24 h repair periods, while no significant difference with Cr unsupplemented cells could be seen at 48 h (Fig. 7B). Importantly, that Cr effects are more pronounced at very early post-challenge times (compare the effect at 0 h with that at 24 h) might suggest that it prevents the induction of the lesions – i.e. a mechanism conceivable with a direct antioxidant activity – rather than acting downstream

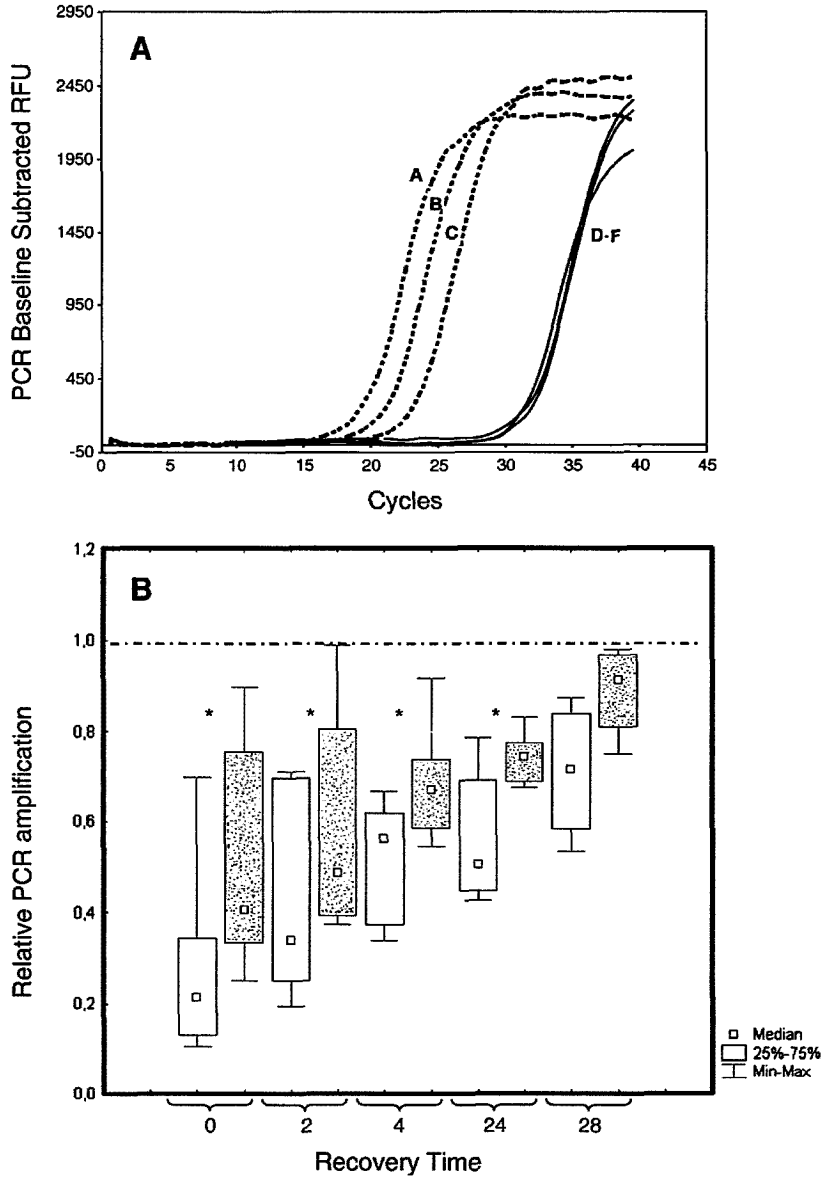

Fig. 7. Effect of Cr preloading on oxidant-induced HUVEC mtDNA damage. (A) Cr-supplemented (10 mM) or unsupplemented cells were treated with 200 μM H_2O_2 for 30 min and immediately assayed for mtDNA damage. Representative amplification curves of Real-time mtDNA PCR are shown: curves A–C were obtained with previous long PCR amplification of 9.129 kb mtDNA: curve A, control DNA; curve B, H_2O_2 + 10 mM Cr-treated DNA; curve C, H_2O_2-treated DNA; curves D–F were obtained without previous long PCR amplification. (B) HUVEC cells were pre-incubated for 24 h with 0 (open bar) or 10 mM (closed bar) Cr and then treated for 30 min in Saline A with 200 μM H_2O_2. The cells were harvested immediately (time 0) or allowed to grow in complete medium for the indicated times (Recovery time=hours). mtDNA damage was assessed by combining long PCR and Real-Time PCR. Results are expressed as relative PCR amplification; the decrease in amplification was calculated comparing treated samples to undamaged control (dashed line). The Box & Whisker graph was obtained from at least four independent experiments. * $P<0.05$, Wilcoxon signed-rank test.

their production. Finally, using the same approaches *o*-phenanthroline completely protects both n and mtDNA from oxidative damage while Trolox was ineffective (not shown).

Hydrogen peroxide is known to produce several types of lesions, including 8-OHdG. This specific lesion is not detected with full efficiency by QPCR in that it does not significantly stall the progression of DNA polymerase. In order to overcome this intrinsic limitation of QPCR we employed an ELISA method which uses a monoclonal antibody against 8-OHdG to detect the amount of modified bases [45]. As shown in Fig. 8, challenge with 200 μM H_2O_2 caused a 2.8 fold 8-OHdG increase in HUVEC nDNA (3.1±0.45 ng/mg of DNA vs. 1.12± 0.15); notably and according to the above QPCR and Real-Time PCR results indicating that Cr was not protective on oxidatively-injured nDNA, Cr supplementation failed to decrease the level of 8-OHdG accumulation in nDNA from H_2O_2-treated HUVEC (Fig. 8).

Fast halo assay was performed in order to investigate the Cr effect on nDNA with an unrelated and independent technique sensitive to a broad range of DNA lesions [46] and results are

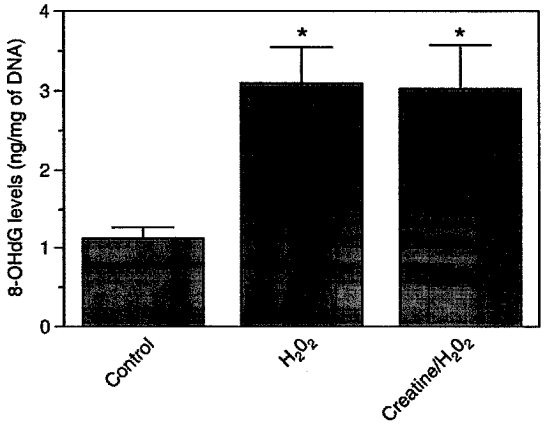

Fig. 8. Determination of 8-OHdG levels by ELISA. HUVEC were preincubated for 24 h with or without 10 mM Cr and then treated for 30 min with 200 mM H_2O_2. Results are expressed as ng of 8-OHdG/mg of DNA. Values represent the means±S.D. (n=3). Student's t -test was used to measure the significance of differences between groups. *P<0.05 control vs. H_2O_2-treated cells or control vs. H_2O_2-treated cells supplemented with 10 mM Cr. P=0.887 H_2O_2-treated cells compared to 10 mM Cr supplemented H_2O_2-treated cells.

shown in Fig. 9. As expected, H_2O_2-treated cells showed significant increase in halo size (see representative micrographs in Fig. 9), which reflects the degree of DNA single strand scission [46]. Pre-incubation of H_2O_2-treated cells with Cr did not show significant protective effect on the extent of nDNA breakage (Fig. 9), which is consistent with QPCR, Real-Time PCR and 8-OHdG data. Notably, according to previously published data [52,53], *o*-phenanthroline completely prevented H_2O_2-induced nDNA damage and, in sharp contrast, Trolox did not (Fig. 9). Finally, it is worth noting that, unlike mtDNA lesions, nDNA breaks induced by H_2O_2 in Cr-supplemented or Cr-unsupplemented cells, were rapidly repaired with identical kinetics ($t_{1/2}$ of approximately 12 min, not shown, see Ref.[54]).

4. Discussion

In this study we investigated the effect of Cr on DNA under oxidative-challenge conditions to understand whether this compound might be considered as a mitochondrially targeted antioxidant which could be helpful in the prevention of ROS-induced mtDNA oxidative damage. Acellular and cellular investigations were carried out.

Acellular assays were performed on circular and linear DNA and Cr protection from oxidative attack was shown. Results obtained from plasmid DNA are in agreement with other data on the assessment of DNA strand breaks obtained using either gel electrophoresis or other techniques that measured the average value of the extent of the lesions [55–60].

Cellular experiments also showed that Cr supplementation exerts genoprotective activity, although limited to mtDNA. The results obtained quantifying the oxidative damage of mtDNA and nDNA from HUVEC cells are consistent with previous researches performed on different human and rodent cell types, showing that mtDNA is more prone to oxidative damage than nuclear DNA [61–63]. In our investigation completion of mtDNA repair was not achieved even after a very long recovery period (48 h) whereas, according to previous observations [54], nDNA-damage was rapidly removed with a $t_{1/2}$ of ca. 12 min (not shown). The persistence of mtDNA lesions suggests that the selected oxidative treatment might have exceeded, at the mitochondrial level, the repair capacity. Indeed, in humans, numerous defence systems protect cellular macromolecules [64] from oxidative challenge, the oxidized DNA is continuously repaired and the oxidized bases excreted into the blood stream [65]. Nevertheless, other deleterious events, such as secondary ROS reactions, take place in the mitochondria and have the potential of overwhelming the mitochondrial repair capacity leading to a vicious cycle of damage which results in persistent mtDNA lesions.

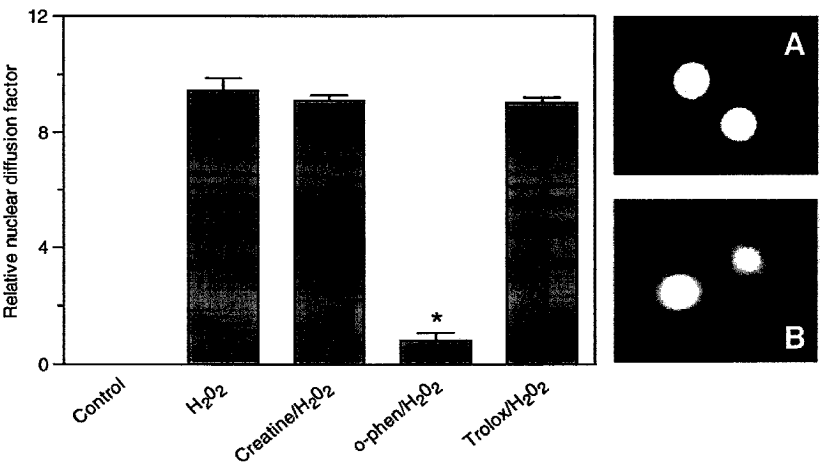

Fig. 9. Effect of Cr on oxidant-induced nDNA damage as assayed with the fast halo assay. HUVEC cells were pre-incubated for 24 h with or without 10 mM Cr, or for 20 min in the absence or presence of 10 μM *o*-phenanthroline or 1 mM Trolox, and then treated for an additional 30 min with 200 μM H_2O_2. nDNA damage was assayed immediately after treatments with the fast halo assay, and the results are expressed as relative nuclear diffusion factor (see Materials and methods). Data are the mean±S.E.M. from three separate experiments, each performed in duplicate. * P<0.001 (unpaired t test) as compared to H_2O_2-alone treated cells. Representative micrographs of HUVEC cells processed for DNA damage with the fast halo assay are also shown in panels A (control cells) and B (H_2O_2-treated cells).

Several studies have documented the powerful protective effects of Cr in oxidative stress associated diseases [18–21] and in neuropathologies, such as Huntington disease, Parkinson disease and amyotrophic lateral sclerosis [66–68]. The novelty of the present work is the finding that Cr protects oxidatively-injured DNA, as shown by both acellular experiments and cell based assays, respectively. In particular, our results show that Cr supplementation significantly protects only mtDNA. That nDNA is not sensitive to Cr-protection (Figs. 6–8) is not surprising. nDNA damage following oxidative stress is usually thought to be a function of site-specific DNA-associated Fe-based Fenton chemistry [69]. It is commonly assumed that iron is normally associated with nDNA and many reports indicate that only iron-chelators, unlike scavenging antioxidants such as Trolox [52,53] (Fig. 9), are capable of protecting cells against oxidative nuclear damage [53,70–72]. Importantly, Cr has been shown to exert its antioxidant, cytoprotective activity *via* a scavenging mechanism rather than through iron-chelation [17]: hence, the Cr lack of protective effects on oxidatively-injured nDNA, as assayed with QPCR, Real-Time PCR, 8-OHdG ELISA and fast halo assay, is not surprising. Conversely our data, as well as others [73–76], imply that mitochondria-accumulating scavenging compounds such as Cr are capable of preventing mtDNA oxidative damage. This differential activity, i.e. mtDNA protection in the absence of nDNA protection, might be due to the different conformation of the two types of DNA. Some indications of this can be observed in acellular experiments on circular and linear DNA (Figs. 2 and 3), which could resemble mitochondrial and nuclear DNA, respectively: plasmidic DNA showed oxidative damages just after 100 min treatment, while a PCR amplification product was damaged after a longer treatment time. Thus it is likely that circular DNA, because of its negative supercoiled structure, is more prone to damage as well as more accessible to Cr and sensitive to its protective effect, than linear DNA. Furthermore, in the specific case of Cr, it should be noted that the Cr-protective effect on mtDNA might be related to its mitochondrial localization. Indeed Cr is actively taken up by specific 55 and 70 kDa mt-Cr transporters [43] into mitochondria, where it is utilized for the energy transport between the site of ATP production and consumption by ATPases. Isolated, respiring mitochondria incubated in 15 mM Cr have been shown to accumulate 20 mM Cr [43]. Notably, under our supplementing conditions, HUVEC intracellular free-Cr level has been shown to be 48.5 nmol/mg of proteins [17], i.e. 8.82 mM (HUVEC volume=5.5 μl/mg of proteins, [77]). As a corollary, the importance of the capacity of an antioxidant to accumulate within mitochondria in order to protect mtDNA from oxidative damage is indirectly emphasized by the observation that Trolox, which is not known to accumulate within these organelles, lacks any protective effect.

Finally, it is worth noting that our results implicitly raise the question of whether oxidative mtDNA and nDNA lesions represent lethal events. Indeed our data confirm the notion that H_2O_2-induced nDNA single strand breaks do not represent a cytotoxically relevant damage [69,78] since in Cr-supplemented H_2O_2-treated cells we observed reduced cytotoxicity in the absence of reduced nDNA breakage. In sharp contrast, we also showed that reduced mtDNA damage is paralleled by a decreased cytotoxic response in H_2O_2-injured Cr-supplemented cells. Thus, it could be inferred that, at least under mild stressing conditions, oxidative mtDNA damage, unlike nDNA damage, represents a cytotoxically relevant type of lesion. However, to answer this important question further studies will be necessary to understand whether this phenomenon is simply incidental or it reflects a causal relationship.

Our study is one of the few reporting the protective effect of Cr on mtDNA. Berneburg et al. [79] demonstrated that 1 mM Cr abolished the induction of mtDNA mutations generated in normal human fibroblast by repetitive UV-A irradiation. In our investigation we used a different cell line, different molecular approaches and a different cellular insult (acute oxidative stress) and we found that mtDNA is protected by Cr.

Data reported by Sestili et al. [17] indicate that Cr affords cytoprotection *via* direct antioxidant capacity with a radical scavenging mechanism: the results presented herein also point to the role of free-Cr as an antioxidant and suggest that the effect of Cr on oxidatively-injured mtDNA might represent an important mechanism contributing to its cytoprotective activity [17] in cells subjected to oxidative stress.

Other antioxidants including vitamins or cofactors such as Coenzyme Q10, ascorbic acid, vitamin E, riboflavin, thiamine, niacin, vitamin K (phylloquinone and menadione), and carnitine have already been used in the treatment of oxidative phosphorylation disorders to increase mitochondrial ATP production and slow or arrest the progression of clinical symptoms [68,80].

Results obtained from cellular experiments suggest that Cr supplementation may play an important role in mitochondrial genome stability in that it could normalize mitochondrial mutagenesis as well as functional consequences such as the decrease of oxygen consumption, mitochondrial membrane potential and ATP content and finally cell survival. Controlling cell life and death, the mitochondria have become the "new cellular brain" and hence represent a new and attractive therapeutic target for the wide range of pathologies where mitochondrial oxidative damage is known to play an etiological role.

On the basis of the results presented herein and because of its biochemical and nutritional features, Cr could be a promising antigenotoxic agent for the treatment of the above-mentioned diseases as well as for the delaying of aging. At this regard, it is worth noting that a very recent and independent study by Bendler et al. [81] has shown that long term Cr supplementation increases health and survival of mice: our results might contribute to the understanding of these important effects.

Further experiments on animal models, clinical and basic research will however be required in order to determine whether Cr antioxidant activity and mtDNA protection against oxidative damage contribute to the reported amelioration [66,67,81] of the symptoms of pathologies related to mtDNA mutations, and future research should also investigate whether long-term Cr supplementation is safe and may be used as a long-term supplement in the prevention of mtDNA damage.

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Elevation of creatine in resting and exercised muscle of normal subjects by creatine supplementation

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1. The present study was undertaken to test whether creatine given as a supplement to normal subjects was absorbed, and if continued resulted in an increase in the total creatine pool in muscle. An additional effect of exercise upon uptake into muscle was also investigated.
2. Low doses (1 g of creatine monohydrate or less in water) produced only a modest rise in the plasma creatine concentration, whereas 5 g resulted in a mean peak after 1 h of 795 (SD 104) μmol/l in three subjects weighing 76–87 kg. Repeated dosing with 5 g every 2 h sustained the plasma concentration at around 1000 μmol/l. A single 5 g dose corresponds to the creatine content of 1.1 kg of fresh, uncooked steak.
3. Supplementation with 5 g of creatine monohydrate, four or six times a day for 2 or more days resulted in a significant increase in the total creatine content of the quadriceps femoris muscle measured in 17 subjects. This was greatest in subjects with a low initial total creatine content and the effect was to raise the content in these subjects closer to the upper limit of the normal range. In some the increase was as much as 50%.
4. Uptake into muscle was greatest during the first 2 days of supplementation accounting for 32% of the dose administered in three subjects receiving 6 × 5 g of creatine monohydrate/day. In these subjects renal excretion was 40, 61 and 68% of the creatine dose over the first 3 days. Approximately 20% or more of the creatine taken up was measured as phosphocreatine. No changes were apparent in the muscle ATP content.
5. No side effects of creatine supplementation were noted.
6. One hour of hard exercise per day using one leg augmented the increase in the total creatine content of the exercised leg, but had no effect in the collateral. In these subjects the mean total creatine content increased from 118.1 (SD 3.0) mmol/kg dry muscle before supplementation to 148.5 (SD 5.2) in the control leg, and to 162.2 (SD 12.5) in the exercised leg. Supplementation and exercise resulted in a total creatine content in one subject of 182.8 mmol/kg dry muscle, of which 112.0 mmol/kg dry muscle was in the form of phosphocreatine.

INTRODUCTION

Creatine (Cr) is one of eight naturally occurring guanidine-derived compounds, which, in their phosphorylated forms [in this case phosphocreatine (PCr)], function in the maintenance of cellular ATP homoeostasis [1, 2]. Cr and its associated phosphotransferase, phosphocreatine kinase, represent a functional improvement over other guanidine phosphagens as judged by its ability to support higher ATP/ADP ratios at equilibrium [1]. It is the only guanidine phosphagen found in higher animals and occurs in highest concentrations in skeletal muscle, with lesser amounts in cardiac and smooth muscle, brain, kidney and spermatozoa. In human skeletal muscle, typified by the quadriceps femoris, a mean content of total creatine (TCr = PCr + Cr) of 124.4 (SD 11.2) mmol/kg dry muscle (DM) was recorded in a study of 81 normal subjects [3]. Similar values have been recorded in other muscles and in other species.

The importance of the Cr phosphagen system to muscle contraction, first demonstrated by Davies [4], resides in its ability to maintain a high intracellular ATP/ADP ratio [5]. This is achieved first through the accumulation of PCr itself which is available as an immediate buffer to ATP use, and secondly by the facilitation of energy translocation from mitochondria to sites of ATP utilization [6–8]. In the context of exercise the availability of PCr has frequently been cited as limiting to the continuation of maximal physical effort. Certainly, the depletion of the muscle PCr store during intensive exercise is commonly associated with the onset of muscle fatigue (e.g. [9]). Utilization of PCr will further contribute to the buffering of H^+, which again will be important to the continuation of maximal exercise.

Despite the central role played by the Cr phosphagen system in energy provision in muscle and other tissues, relatively little is known concerning its

Key words: creatine, exercise, muscle, phosphocreatine.
Abbreviations: Cr, creatine; $Cr.H_2O$, creatine monohydrate; DM, dry muscle; PCr, phosphocreatine; TCr, total creatine.
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uptake and the regulation of the TCr pool. Biosynthesis in mammals is restricted to just a few tissues, principally liver, pancreas and kidney [10], but may be augmented in meat eaters by small amounts of Cr in the diet [11]. Whatever the source, Cr must be concentrated from plasma into tissues against gradients which, for skeletal muscle, may approach 200:1. Oral administration of analogues of Cr have been shown to inhibit Cr uptake [12] and over time will replace Cr in muscle, causing a fall in PCr content [13–15].

There have been few reports of the effect of Cr supplementation and in none of these has direct measurement of the tissue content of TCr been made. From isotope-dilution studies using ^{15}N-labelled Cr and measurements of creatinine excretion in subjects fed 10 g of Cr/day, Crim *et al.* [16] concluded that the body pool was influenced by dietary supply. Cr has been administered at a dose of 1.5 g daily for 1 year to patients with gyrate atrophy of the choroid and retina with clinical success [17], while low-dose preparations are available for use by athletes.

The object of the present study was to test whether supplementary Cr added to the diet was first absorbed and secondly, when continued over a period of time, resulted in an increase in the muscle content. The results indicated that this was achieved within a few days and that a further increase in the muscle store was possible when supplementation was combined with regular exercise.

METHODS

Subjects

Permission to undertake an investigation of Cr uptake into muscle was first obtained from the ethical committee of the Karolinska Institute.

Seventeen subjects (five females and 12 males) aged between 20 and 62 years participated in the study. Subjects varied greatly in their level of fitness, although this was not quantified in any way. During the study, subjects continued their normal pattern of life and no restraints were imposed upon them in terms of diet or general activities. Two subjects, nos. 13 and 16, were vegetarians. The nature of the investigation and possible risks involved were explained to each subject before their consent to participate was obtained.

Subjects underwent a basic medical examination and blood samples were taken for haematological examination and screening of coagulation parameters and bilirubin.

Experimental protocol

Preliminary studies were undertaken (see the Results section) to establish a suitable dose of creatine monohydrate ($Cr.H_2O$). That chosen was 5 g of $Cr.H_2O$ (33.6 mmol) which was easily dissolved in 300 ml of warm-to-hot water with no detectable formation of creatinine. A dose rate of four times per day was established using subjects nos. 1 and 2, although this was later increased to six times per day in others.

$Cr.H_2O$ was administered to 12 subjects according to the following schedule: 4×5 g was taken per day for 4.5 days (subject nos. 1 and 2), 7 days (subject nos. 3 and 4) and 10 days (subject no. 5), and 6×5 g was taken for 7 days (subject nos. 6–8) and on alternate days for 21 days (subject nos. 9–12).

To study any interaction between exercise and Cr uptake, a further five subjects were given supplementary Cr, but in this case each performed 1 h of bicycle ergometer exercise on each day using one leg. The other leg was rested during this time and served as a control. Subjects were allowed to adjust the work intensity themselves, but were asked to undertake the most that they could achieve in the 1 h. Cr feeding protocols for these five subjects were as follows: 4×5 g was taken for 3.5 days [subject no. 1 repeated (1R)], and 6×5 g was taken for 4 days (subject nos. 13–15) and 7 days (subject no. 16).

Muscle samples

In all cases a single muscle biopsy of the vastus lateralis of the left or right leg was taken in the morning before the start of Cr supplementation, using a 6 mm Bergström–Stille biopsy needle [18]. Biopsy samples were snap-frozen in liquid nitrogen, freeze-dried, powdered and analysed for PCr, Cr and ATP [3]. From previous work, a coefficient of variation in the resting contents of each of these metabolites, and of TCr, between different sampling sites on the same muscle of 5% or less was assumed [3].

In the investigation of the effects of exercise, only one biopsy was initially taken. In two subjects this was from the leg which was subsequently exercised, and in the other three it was from the 'control' leg. Results from this biopsy were considered to describe the contents of ATP, PCr and TCr in both legs. Earlier work has again demonstrated a coefficient of variation in the resting contents of each of these between comparable sampling sites on collateral legs of 5% or less. This estimate was the same whether legs were classified as left or right, or dominant and non-dominant. Subsequent biopsies in this part of the study were taken from both legs.

Blood samples

Venous blood samples (5 ml) were taken from the left or right cubital vein using lithium heparin as anticoagulant. One sample was taken before and one after Cr supplementation for routine haematology and plasma biochemistry. Cr was determined in neutralized perchloric acid extracts using essentially the same method as for muscle. Creatinine

was determined using a method developed for use on the Kodak autoanalyser, (Rochester, NY, U.S.A.). In some early studies, Cr and creatinine were determined colorimetrically using an enzymic method (Wako Chemicals GmbH, Neuss, Germany). Plasma samples were stored frozen at −85°C until analysed 2–3 days later, to minimize conversion of Cr to creatinine.

Urine samples

Twenty-four hour urine samples were collected from most subjects for analysis of Cr. This was performed on a neutralized perchloric extract by the same method as for muscle.

RESULTS

Selection of the Cr dose

Preliminary studies indicated that oral consumption of a solution of $Cr.H_2O$ resulted in a rapid rise in the plasma Cr concentration. As a result we decided to find a dose which increased the plasma concentration to at least 500 μmol/1. Doses of the order of 1 g of $Cr.H_2O$ were quickly discounted, since peak concentrations rarely exceeded 100 μmol/l. Single doses of 5 g on the other hand resulted in peak concentrations of 690–1000 μmol/l. Fig. 1 shows the results from three subjects where plasma samples were taken 0–7 h after ingestion of 5 g of $Cr.H_2O$. In these a mean peak concentration of 795 (SD 104) μmol/l was observed 1 h after dosing. Repeated dosing with 5 g of $Cr.H_2O$ every 2 h over 8 h maintained a high plasma Cr concentration (in several subjects in excess of 1000 μmol/l) for a major part of this time. Despite this the plasma creatinine concentration was not increased, remaining close to 70–100 μmol/l.

On the basis of these results, a 5 g dose was adopted for the supplementation studies. This is approximately equal to the TCr content of 1.1 kg of fresh, uncooked steak.

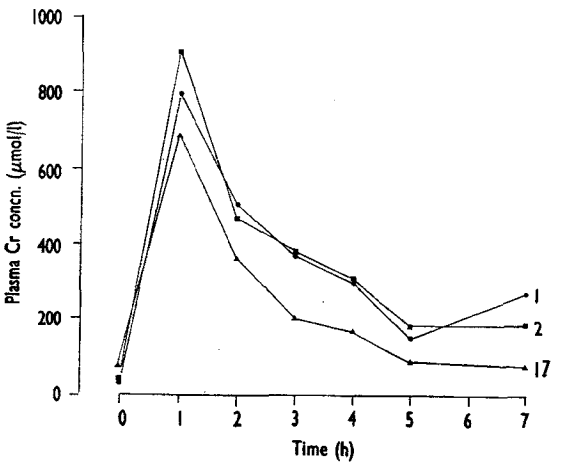

Fig. 1. Concentration of Cr in the plasma of three subjects after a single dose of 5 g of $Cr.H_2O$ dissolved in 300 ml of warm water administered at 0 h. Subjects were aged 28–62 years and had body weights of 76 kg (subject no. 1), 83 kg (subject no. 17) and 87 kg (subject no. 2).

Effect on muscle TCr content

The effects of prolonged supplementation with $Cr.H_2O$ are summarized in Fig. 2. Initially, studies were carried out on two subject taking 4 × 5 g of $Cr.H_2O$ for 4–5 days. This resulted in an increase in the TCr pool in excess of 20%. No ill effects were noted and blood profiles remained normal. Longer durations and more frequent dosing were tried (up to 6 × 5 g per day) and finally one group of subjects was administered 6 × 5 g of $Cr.H_2O$ on alternate days, for 21 days.

Results are also included in Fig. 2 from the control leg of those subjects (nos. 1R and 13–16), who exercised for 1 h each day with the collateral leg. Changes in muscle TCr content in the control legs of these subjects were comparable with the changes in subjects engaged in normal activity.

The mean TCr content before Cr feeding of all

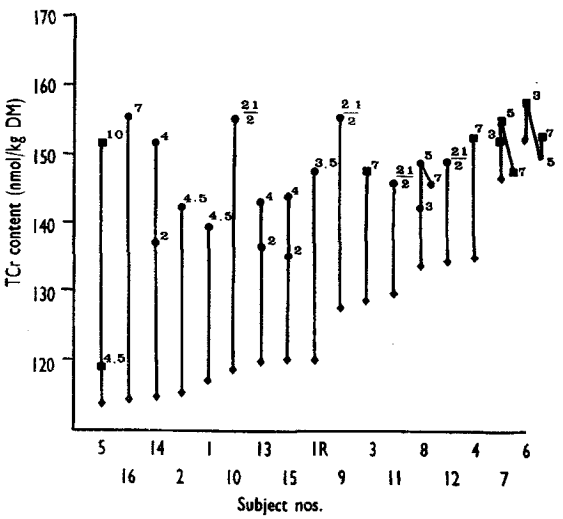

Fig. 2. TCr content of the quadriceps femoris before (◆) and after (■, ●) supplementation with $Cr.H_2O$. Supplementation rates were as follows: 4 × 5 g for 4.5 days (subject nos. 1 and 2), 7 days (subject nos. 3 and 4) and 10 days (subject nos. 5); 6 × 5 g for 7 days (subject nos. 6–8) with biopsies on days 3, 5 and 7, and on alternate days for 21 days (subject nos. 9–12). Also included are the results from the control leg of five subjects who performed 1 h of strenuous exercise per day with the collateral leg. Supplementation rates were in this case: 4 × 5 g for 3.5 days (subject no 1R); 6 × 5 g for 4 days (subject nos. 13–15) with biopsies on days 2 and 4, and 7 days (subject no. 16). Subjects have been arranged in order of increasing initial TCr content. Numbers on the Figure denote the days of supplementation at the time of the biopsy, ■, Female subjects; ●, male subjects.

subjects was 126.8 (SD 11.7) mmol/kg DM, close to a value of 124.4 (SD 11.2) mmol/kg DM observed earlier in 81 normal subjects [3]. With the exception of two subjects with an initial TCr content above 145 mmol/kg DM, all others showed an increase with Cr supplementation. In these the final TCr content was in every case >140 mmol/kg DM and in six subjects this even exceeded 150 mmol/kg DM. The mean TCr content in all subjects after Cr supplementation was 148.6 (SD 5.0) mmol/kg DM. The increases in TCr observed appeared less dependent upon the duration of supplementation and daily dose rate than upon the initial TCr content, i.e. the greatest increases occurred in subjects with the lowest initial content. These data suggest that 155 mmol/kg DM may represent an upper limit for the TCr pool, at least when using dose regimens of 4–6 × 5 g per day.

Again no ill effects of Cr supplementation were noted and there were no changes in blood profiles.

The rise in the TCr pool resulted from increases in both PCr content and Cr content. Before feeding mean PCr content was 84.2 (SD 7.3) mmol/kg DM, or 66.8 (SD 3.9)% of the TCr content. After Cr supplementation, the mean PCr content was 90.6 (SD 4.8) mmol/kg DM, or 61.0 (SD 2.9)% of the TCr content. The largest increase in PCr content, from 76.7 to 100.0 mmol/kg DM, was shown by subject no. 14.

There was no increase in ATP content in muscle associated with the increase in PCr content. Focusing on the first 10 subjects in Fig. 2 (i.e. nos. 5–9), which as a group showed a mean increase in TCr content of 30.3 (SD 6.9) mmol/kg DM, the mean ATP contents before and after supplementation were 24.9 (SD 1.9) and 25.8 (SD 1.2) mmol/kg DM, respectively. The difference in contents was not statistically significant ($P > 0.05$). Fig. 3 shows the change in TCr content relative to ATP content. This is possibly a better representation of the change in TCr content with Cr supplementation, than that shown in Fig. 2, since it cancels out variance in contents due to differences between biopsy samples in their contents of blood and connective tissue. Expressed in this form, Fig. 3 indicates that some increase in TCr content probably occurred in all subjects, including nos. 6 and 7 (compare with Fig. 2).

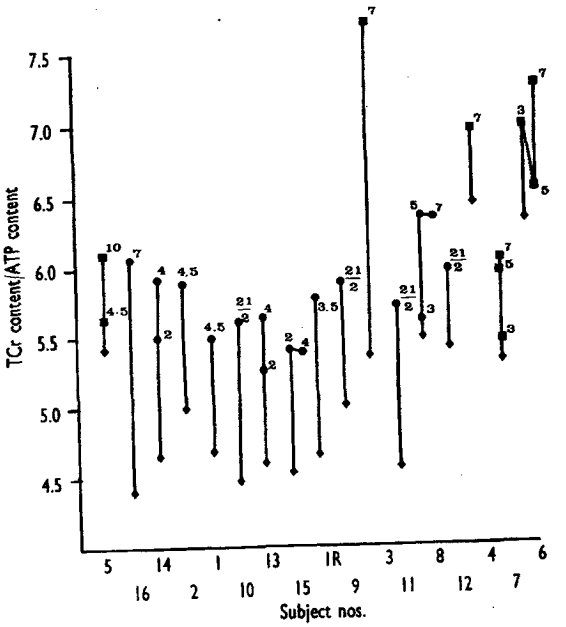

Fig. 3. Ratio of TCr content to ATP content in biopsy samples of the quadriceps femoris before (◆) and after (■, ●) supplementation with $Cr.H_2O$. Details of the doses are given in the legend to Fig. 2. Numbers on the Figure denote the days of supplementation at the time of the biopsy. ■, Female subjects; ●, male subjects.

Exercise and Cr supplementation

The increase in muscle TCr content in the control leg of subjects performing one-legged exercise was comparable with that shown by subjects engaged only in normal activity. Exercise, however, resulted in a significantly ($P < 0.05$) greater increase in TCr content with Cr supplementation (Fig. 4, exercised leg). In these five subjects the mean TCr content increased in the control leg from 118.1 (SD 3.0) mmol/kg DM to 148.5 (SD 5.2) mmol/kg DM and in the exercised leg to 162.2 (SD 12.5) mmol/kg DM. In four of the subjects the TCr content exceeded 155 mmol/kg DM in the exercised leg and in subject no. 16 it reached 182.8 mmol/kg DM.

The PCr content at the end of supplementation in the exercised legs averaged 103. 1 (SD 6.2) mmol/kg DM compared with 93.8 (SD 4.0) mmol/kg DM in the control legs and 81.9 (SD 5.6) mmol/kg DM before supplementation. The highest PCr content recorded, 112.0 mmol/kg DM in the exercised leg compared with 79.8 mmol/kg DM before supplementation, was in subject no. 16.

As before, muscle ATP content was unaffected by the changes in TCr content. In these five subjects, the ATP content before supplementation was 25.9 (SD 0.8) mmol/kg DM and after supplementation it was 25.1 (SD 1.7) mmol/kg DM in the control legs and 24.3 (SD 2.0) mmol/kg DM in the exercised legs. Fig. 5 shows the changes in TCr content relative to ATP content. Presented in this form, the results imply that the apparent increase in the TCr content in the exercised legs of subject nos. 1R and 14 may have been underestimated in Fig. 4, probably for the reasons discussed earlier. From an initial mean TCr/ATP ratio of 4.57 (SD 0.13), TCr/ATP ratios in the exercised legs exceeded 6.0 in all subjects and in two exceeded 7.0. High values had been seen previously in subject nos. 3, 4 and 6 (Fig. 3), each of which had shown high TCr contents before supplementation.

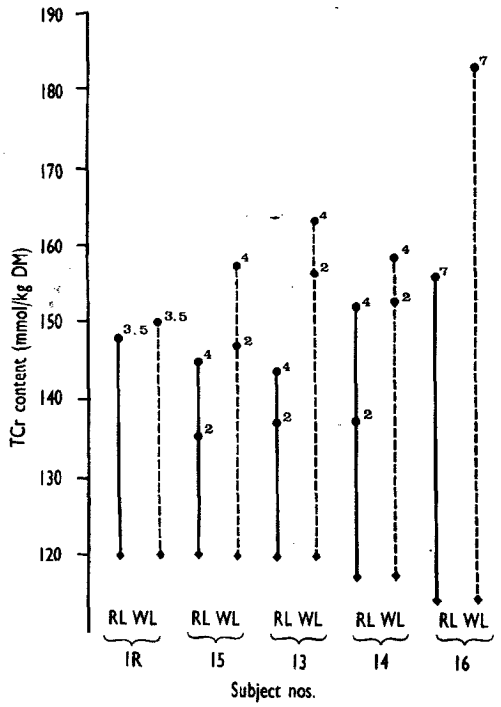

Fig. 4. Effect of exercise and Cr supplementation on the TCr content of the quadriceps femoris. During the period of supplementation subjects performed 1 h of strenuous exercise on a bicycle ergometer using one leg only (work leg = WL). During this time the control leg was rested (rest leg = RL). For the rest of the time the subjects went about their normal daily activities. Doses rates of $Cr.H_2O$ used were: 4×5 g for 3.5 days (subject no. 1R); 6×5 g for 4 days (subject nos. 13–15) with biopsies on days 2 and 4, and 7 days (subject no. 16). To minimize the number of biopsies taken, only one was taken before supplementation. This was from the rest leg and is assumed to describe also the pre-supplementation TCr content in the collateral leg. Subjects have been arranged in order of increasing initial TCr content. All subjects were males. Numbers on the Figure denote the days of supplementation at the time of the biopsy. ◆, Before supplementation; ●, after supplementation.

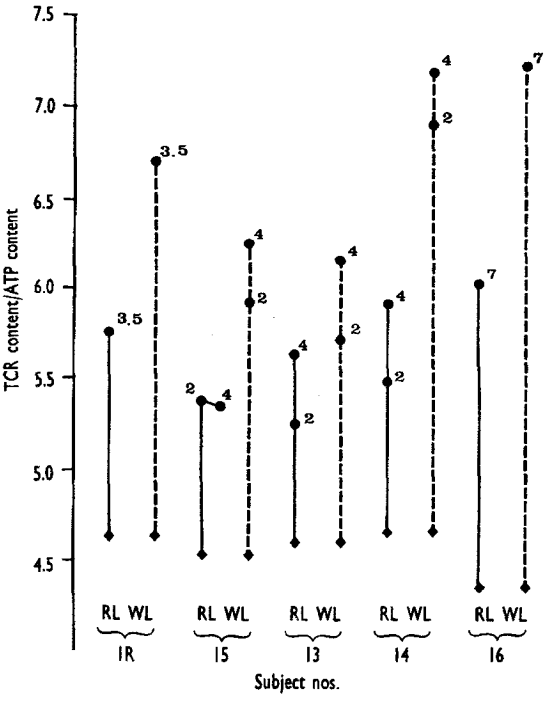

Fig. 5. Ratio of TCr content to ATP content in biopsy samples of the quadriceps femoris before (◆) and after (●) supplementation with $Cr.H_2O$ in subjects performing an additional 1 h of strenuous exercise per day. Abbreviations: RL, rest leg; WL, work leg. Details of the doses are given in the legend to Fig. 4. Numbers on the Figure denote the days of supplementation at the time of the biopsy.

Urine excretion

Although 24 h urine samples were collected from all subjects, this was mainly intended for monitoring purposes and only in four subjects were complete data obtained. These are presented in Table 1. These and the less complete records all showed the same pattern with the greatest uptake of Cr occurring during the first 2 days. For subject nos. 13–15, maintained on the same supplementation protocol, Cr excretion by the kidney over the first 3 days accounted for 40 (SD 14)%, 61 (SD 3)% and 68 (SD 15)% of the dose administered, respectively. Greater uptake during the first 2 days was further indicated by the estimated increase in muscle TCr content during this time. The estimated mean uptake of Cr during the first 2 days in these three subjects was 17.7 (SD 3.4) g or approximately 32% of the dose administered. From day 2 to day 4, further uptake amounted to 9.1 (SD 1.6) g of Cr.

DISCUSSION

Despite the obvious importance of the Cr phosphagen system to cell viability, surprisingly little is known concerning the metabolism of Cr in the intact organism. Biosynthesis from arginine and glycine as precursors is clearly adequate for maintenance purposes in the normal individual. This is demonstrated by the present results, where two of the subjects (nos. 13 and 16) were vegetarians. Despite the lack of dietary Cr, the initial TCr contents (120.0 and 114.6 mmol/kg DM) in these two subjects were within the normal range. Vegetarians have been shown to have lower Cr and creatinine concentrations in serum and a decreased output in urine [19], but this does not necessarily imply decreased tissue contents. Based on measurements of renal excretion of creatinine, the daily requirement for Cr supplied through the diet or from endogenous synthesis, in a 70 kg man, is approximately 2 g/day [10].

With impairment of Cr biosynthesis, as described for patients with gyrate atrophy of the choroid and retina, plasma concentrations and renal excretion are again decreased and in this case probably indicate a decreased body TCr content [20]. As far

Table 1. Measurements of Cr output in the urine (*B*) together with estimates of the maximum amount of the dose on each day (*A*) which may have been retained (*C*) in comparison with the amount calculated from the increases in TCr to be accumulated in muscle (*D*). Data are from four subjects supplemented with 20 g of $Cr.H_2O$ (17.6 g of Cr) or 30 g of $Cr.H_2O$ (26.4 g of Cr) per day. In estimating Cr uptake into muscle (D), the amount of muscle was estimated as 40% of body weight. To convert this to DM, a water content of 3.3 litres/4.3 kg wet weight was assumed. For subject nos. 13–15, who undertook one-legged exercise, the average change in the musculature as a whole was estimated from: (80% of the increase in TCr content in the control leg) + (20% of the increase in TCr content in the exercised leg). For subject nos. 14 and 15, urine collection was stopped prematurely before intake of the full dose of 26.4 g of Cr. Thus 13.2 g of Cr is recorded as the dose taken for that day up to the time of the final urine collection, although this should be 26.4 g when considering the changes in muscle.

Day	A Dose (g)	B 24h urinary excretion (g)	C (A)–(B) (g)	D Muscle content (g)	A Dose (g)	B 24h urinary excretion (g)	C (A)–(B) (g)	D Muscle content (g)
	Subject no. 5				Subject no. 13			
1	17.6	12.6	5.0		26.4	13.0	13.4	
2	17.6	15.3	2.3		26.4	16.9	9.5	
								16.4
3	17.6	14.7	2.9		26.4	22.1	4.3	
4	17.6	16.0	1.6		26.4	22.0	4.4	
				13.5				24.5
5	17.6	15.8	1.8					
	Subject no. 14				Subject no. 15			
1	26.4	6.2	20.2		26.4	12.1	14.3	
2	26.4	15.4	11.0		26.4	15.9	10.5	
				21.6				15.2
3	26.4	14.0	12.4		26.4	17.6	8.8	
4	13.2	9.0	4.2		13.2	8.9	4.3	
	(26.4)			32.5	(26.4)			23.4

as we are aware, there have been no systematic studies of the effects on Cr homoeostasis of diseases affecting the major organs of biosynthesis.

For most individuals, Cr is a normal constituent of the diet, providing an estimated intake of up to 1 g/day [11]. In carnivores, dietary intake of Cr will be very much greater and on a g/kg body weight basis will approach the amounts administered in the present study.

The total doses of $Cr.H_2O$ administered in the present study varied from 70 g (61.5 g of Cr) given over 3.5 days (subject no. 1R) to 330 g (290 g of Cr) over 21 days (subject nos. 9–12). This compares with an estimated total body pool of TCr of 120 g in a 70 kg man. Uptake appeared greatest during the first days of supplementation and, as stated, amounted to a mean of 32% of the dose administered during the first 2 days to subject nos. 13–15 (Table 1). In these subjects renal excretion over days 1, 2 and 3 amounted to 40%, 61% and 68%, respectively.

No attempt was made in this study to standardize the dose given on the basis of body weight. Consequently, for the subjects involved, the single 5 g dose of $Cr.H_2O$ represented a range of 50 (subject no. 2) to 90 (subject. no. 5) mg of Cr/kg body weight. In selecting the 5 g dose our aim was to provide sufficient Cr to raise the plasma concentration to a peak of 500 μmol/l or more, even in higher-weight individuals. Cr entry into muscle occurs via a saturable process [15], which in rat extensor digitorum longus muscle exhibits a K_m of 500 μmol/l [12]. (No equivalent data is available for human muscle.) We reasoned on the basis of this that plasma concentrations of this order would have to be aimed for to effect a measurable increase in TCr over a relatively short period. As shown in Fig. 1, this was achieved, although because of the short half-life of Cr in plasma (1–1.5 h), the concentration soon fell below the 500 μmol/l level, at least after a single dose.

The effect of supplementation was greatest in those subjects with the lowest initial TCr contents and had little effect in those where the initial content was close to the upper end of the normal range (Fig. 2). The greatest uptake appeared to occur during the first 2 days and at the end of supplementation all subjects lay within a narrow band of 140–160 mmol of TCr/kg DM, halving the between-subject variance. Contents of 150–160 mmol of TCr/kg DM have occasionally been observed in normal subjects [3, 21]. Whether further increases would have occurred with higher or more frequent doses, or if supplementation had been further prolonged, is not known. In the limited number of subjects studied there were no apparent effects of age or sex. It has recently been shown by Forsberg *et al.* [22] that females have a higher TCr content than males, but no effect of age was found in this study. Of the two vegetarians, (subject no. 16), showed the largest increase of all subjects in TCr content (+40.9 mmol/kg DM), although a more average response was shown by the other (subject no. 13).

Expression of the results as the TCr/ATP ratio (Fig. 3) helps to identify errors arising from variation in the blood and connective tissue contents of biopsy samples, since the ATP content itself did not change. It also provides a measure of the number of molecules of Cr+PCr in support of each ATP. All subjects, including those with the highest initial TCr contents, showed an increase, which in subject no. 3 was inexplicably much greater than in the others.

Twenty to forty per cent of the increase in TCr content was accounted for by PCr. However, because of damage inflicted during muscle biopsying [23] it is possible that the increase *in situ* in PCr content was greater.

As shown in Figs. 4 and 5, one-legged exercise increased the uptake of Cr locally but had little or no effect in the collateral leg. Although the effect of exercise without supplementation was not investigated in this study, previous work has shown that the muscle TCr store is unchanged by 8 weeks of intensive training at work rates comparable with those employed here [24, 25]. Judged from the difference in TCr contents, Cr uptake was enhanced 54 (SD 25)% by 1 h of exercise/day. [An increase of 84 (SD 22)% is indicated by the TCr/ATP ratios.] As noted earlier, the greatest increase in TCr was in subject no. 16, a vegetarian. It is possible that the increase in Cr uptake resulted from the increase in total blood flow to the exercised muscle, although a change in the transport kinetics of Cr across the fibre membranes is also possible.

Because of the central role played by PCr in the maintenance of ATP homoeostasis and in energy transport, the present results are likely to be of interest in a wide variety of physiological conditions. In this study we have focused only upon uptake into skeletal muscle, but similar effects may be anticipated in other Cr-containing tissues, e.g. heart muscle, brain, macrophages and spermatoza. Although PCr is most commonly associated with ATP used during muscle contraction, it has a much wider role in the provision of high-energy phosphate to sites of ATP utilization [8].

We believe that the present results will be of interest to those concerned with athletic performance, especially if it is subsequently shown that increase in TCr content leads to an improvement in work capacity. Significantly, Sipilä *et al.* [17] in their study of patients with gyrate atrophy of the choroid and retina treated with 1.5 g of supplementary Cr/day, recorded that patients had an impression of increasing strength, and also that one, a runner, beat his former 100m record.

The use of Cr supplementation to increase its content in muscle evokes an earlier report from this laboratory in which procedures were described for increasing the muscle glycogen stores [26]. This procedure was subsequently adopted by many athletes as part of their general preparation for competition.

ACKNOWLEDGMENTS

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CREATINE KINASE ELEVATIONS AND AGGRESSIVE BEHAVIOR IN HOSPITALIZED FORENSIC PATIENTS

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The relationship between creatine kinase (CK) and aggressive behavior was tested in 195 males consecutively admitted to a forensic hospital. Among patients receiving antipsychotic medication, the most violent patients had higher CK levels than less violent patients. This was not the case in patients who did not receive antipsychotic medication. CK levels were not influenced by age, ethnicity, or clinical diagnosis. CK levels were however influenced by prior assaultiveness and restraints. When these two factors were controlled for, CK levels remained strongly associated with subsequent violence. CK appears to be a potential predictor of violent behavior. It has the advantage of easy availability

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in comparison to other biological markers of aggression (e.g., 5-HIAA). Prospective studies are needed to confirm the validity of this biobehavioral association.

Numerous studies have reported elevated levels of serum creatine kinase (CK), the enzyme previously known as creatine phosphokinase (CPK), in psychotic patients. These elevations have been noted among patients with schizophrenic and affective psychoses and some of their first-degree relatives (1). Of the three isoenzymes of CK (CK-MM, CK-MB and CK-BB, mostly found in skeletal muscles, the heart and the brain, respectively), it is the CK-MM isoenzyme that is present in psychotic patients in elevations ranging from a few percent above the upper limit of the normal range (15-120 U/l) (2) to five thousand percent above that limit (1, 3). These abnormalities in CK activity consist of transient elevations in CK levels (lasting from a day to a month) as well as elevations in mean CK levels, i.e., CK levels averaged over time (4). In some patients these elevations are attributable to factors such as physical trauma, intramuscular injections, restraints, neuroleptic malignant syndrome, acute dystonic reaction, alcoholism, thyroid disease, cardiac disease, seizure disorder, recent electroconvulsant therapy, and increased motor activity (4), but in many patients CK elevations cannot be attributed to these factors (3).

The biological importance of CK elevations without known etiology may be related to abnormalities of muscle tissue. For example, muscle biopsies reveal abnormal morphology in nearly half of acutely psychotic patients (5-7). This could be due to a defect in the neurotrophic influence on muscle cell membrane integrity leading to increased release of CK from muscle, or to the independent effects of a gene causing pleiotropic action (1). A correlation between CK abnormalities in psychotic patients and their unaffected relatives also supports the possibility of a genetic defect.

Two studies have suggested that aggressive behavior and affect are associated with serum CK elevations. Meltzer (8) reported that in patients with acute psychosis, elevated ratings of excitement and anger correlated with CK elevations. Isaacs and Yesavage (9) found that CK levels were positively associated with

violence prior to admission to an inpatient unit and to seclusion for dangerous behavior, but not to violence during hospitalization. We investigated the hypothesis that aggressive behavior may be associated with serum CK elevations in a sample of violent forensic patients.

METHODS

Subjects were 195 consecutive male admissions to the Whiting Forensic Division of Connecticut Valley Hospital. They participated in studies of aggressive behavior that have been described elsewhere (10-14). Whiting is a JCAHO-accredited, HCFA-certified, maximum-security state facility dedicated to the evaluation and treatment of forensic patients. In the overall sample, patients were referred to WFI based on: insanity acquittals (31%), court-ordered evaluations (mostly evaluations of competency to stand trial, 16%), acute illness of correctional inmates (29%), or civil commitments (24%). Patients ranged in age from 18 to 67 years, with a mean of 28.7 years and a standard deviation of 9.5 years. Fifty-three percent were White, 36% Black, and 11% Hispanic. The study was performed retrospectively by using existing archival data. Informed consent was thus not required (15).

Within 24 hours following admission, most patients received a physical examination. Their blood was drawn for routine laboratory analysis including determination of serum CK level. A minority of patients refused the blood drawing or were too agitated to cooperate. Of the initial pool of subjects, 164 subjects had complete admission physical examination and blood work including serum CK levels.

The aggressive behavior of all subjects was continuously monitored in the hospital-wide aggressive behavior monitoring system using the Overt Aggression Scale (OAS), a scale widely used in the assessment of inpatient violence (11, 16-17). The OAS uses nursing staff observations to categorize instances of aggressive behavior into four categories (verbal aggression, physical aggression against objects, self, or others) each divided into 4 severity levels. For instance, in the verbal aggression category, behaviors such as "patient makes loud noises, shouts angrily" are assigned

a severity rating of 1, whereas behaviors such as "patient makes clear verbal threats of violence towards others, or requests help to control self" are assigned a severity rating of 4. The OAS yields several summary scores for individual patients, including a mean severity score (the average level of severity of aggressive behavior over a given period of time, on a scale ranging from 1 to 16), a frequency score (the total number of aggressive incidents over a given period of time regardless of type or severity) and an aggression type score [the average level over time, on a scale ranging from 1 (verbal aggression) to 4 (physical aggression against others)]. Reliability and validity of the OAS in inpatient settings have been found to be high (18-20).

To test the hypothesis that CK is positively associated with subsequent violent behavior, the relationship between admission serum CK levels and subsequent aggressive behavior was examined. A median split procedure followed by two-tailed *t*-tests were performed on the OAS variables mean severity of aggression and frequency of aggression in order to compare patients exhibiting high vs. low levels of aggression. To examine the relationship between CK and verbal vs. physical type of aggression, patients who engaged only in verbal aggression were compared to patients who engaged in physical aggression using a two-tailed *t*-test.

T-tests and subsequent factorial analyses of variance were conducted to examine the influence of other factors that have been shown to be related to CK elevations. These were: diagnosis (resulting from independent evaluations by a board certified senior psychiatrist and senior clinical psychologist using interviews, psychological/neuropsychological assessments, social histories, neurological assessments, and records of past hospitalizations); antipsychotic medication (administered orally and intramuscularly); agitation/hyperactivity (rated on a three-point scale by two independent raters; $r = .86$); neuroleptic-induced extrapyramidal symptoms (EPS); accidents (e.g., falls); physical exercise; muscular hypertonicity (reported on the admission physical); assaultiveness prior to admission; and the use of restraints prior to admission (and hence, prior to CK determination). To control for multiple comparisons, alpha was set at .005 in these comparisons.

RESULTS

Admission CK as a Predictor of Subsequent Aggression

CK levels differed substantially as a function of severity, frequency, and type of subsequent aggression, with the more aggressive patients having higher CK levels. The more severely aggressive patients (whose mean severity scores were greater than 11.6, the median severity score) had higher CK levels than the less severely aggressive patients (whose mean severity scores were less than 11.62; $t = 2.31$, df = 162, $p = .022$). The more frequently aggressive patients (whose mean frequency scores were greater than 5, the median frequency score) had higher CK levels than the less frequently aggressive patients (whose mean frequency scores were below 5; $t = 1.99$, df = 162, $p = .048$). Patients ($n = 82$) whose aggression was exclusively verbal (i.e., who never engaged in physical aggression) had lower CK levels than patients ($n = 82$) who engaged in physical aggression ($t = 2.55$, df = 162, $p = .012$; see Table 1).

Admission CK as a Function of Prior Violence

Patients ($n = 21$) who were in restraints during the seven day period prior to the CK determination had higher CK levels (mean

TABLE 1

Admission CK Levels (U/l) as a Function of Severity, Frequency, and Type of Subsequent Aggression

	Mean CK (SD)	*t**	*p*
High severity of aggression	224.2 (424.0)	2.31	.022
/Low severity of aggression	112.8 (104.5)		
High frequency of aggression	221.7 (446.4)	1.99	.048
/Low frequency of aggression	124.8 (107.3)		
Physical aggression	238.7 (451.7)	2.55	.012
/Verbal aggression	114.9 (107.1)		

*df = 162 in all comparisons.

= 473.4 U/l, SD = 746.8) than patients (n = 143) who were not (mean = 123.7 U/l, SD = 134.2) (t = 5.14, df = 162, $p < .001$). Similarly, patients who were assaultive during that time period (n = 17) had higher CK levels (mean = 437.9 U/l, SD = 740.0) than patients (n = 147) who were not (mean = 137.4 U/l, SD = 199.5) (t = 3.91, df = 162, $p < .001$). All patients (n = 17) who had been assaultive seven days prior to the CK measurement had also been placed in restraints. These two variables were thus highly related (r = .77, n = 164, $p < .001$). Because both factors (assaultiveness and being placed in restraints) are known to increase CK levels (2), the more inclusive variable "having been in restraints seven days prior to CK measurement" was used as a between subject factor in subsequent comparisons in order to evaluate its statistical contribution to the link between CK and aggression scores. Alpha was set at .005 to control for multiple comparisons.

A 2 × 2 [high vs. low frequency of aggression (defined through median split) × restraints versus no restraints] analysis of variance (ANOVA) of CK levels revealed main effects for frequency of aggression (F = 9.01, df = 1, 160, p = .003) and for restraints (F = 19.54, df = 1, 160, $p < .001$). An interaction of these two factors approached conventional levels of significance (F = 7.15, df = 1, 160, p = .008). A 2 × 2 [high vs. low severity of aggression (defined through median split) × restraints versus no restraints] ANOVA of CK levels revealed main effects for severity of aggression (F = 17.51, df = 1, 160, $p < .001$) and restraints (F = 14.27, df = 1, 160, $p < .001$), and an interaction of these two factors (F = 14.93, df = 1, 160, $p < .001$). A 2 × 2 [verbal versus physical aggression × restraints versus no restraints] ANOVA of CK scores revealed main effects for type of aggression (F = 18.42, df = 1, 160, $p < .001$) and for restraints (F = 22.34, df = 1, 160, $p < .001$), and an interaction between these factors (F = 13.92, df = 1, 160, $p < .001$) (see Table 2).

The sample was then divided into patients who did (n = 110) vs. those who did not (n = 54) receive antipsychotic medication during their hospitalization. The aforementioned ANOVAs were repeated in these two subsets of patients using the same approach to control for multiple comparisons (see Table 3). All main effects and two out of three interactions were significant among patients

TABLE 2

Admission CK Level (Mean and SD) as a Function of Prior Restraints and Frequency, Severity, and Type of Subsequent Aggression

		Prior Restraints	
		Absent	*Present*
Subsequent aggression:			
Frequency of aggression:	Low	114.2 (87.2)	233.5 (208.4)
	High	136.6 (179.1)	621.1 (917.7)
Severity of aggression:	Low	113.3 (105.1)	107.4 (105.6)
	High	135.2 (160.4)	656.4 (863.0)
Type of aggression:	Verbal	108.6 (94.3)	173.7 (187.9)
	Physical	145.3 (174.9)	698.3 (927.2)

All main effects (restraints, frequency of aggression, severity of aggression, type of aggression) were significant ($p < .005$). The interactions between restraints and severity of aggression ($p < .001$) and between restraints and type of aggression ($p < .001$) were significant. The interaction between restraints and frequency of aggression approached significance ($p = .008$, with alpha = .005).

receiving antipsychotic medication; the interaction of frequency of aggression and restraints approached significance ($F = 7.05$, df = 1, 106, $p = .009$). Among patients receiving no antipsychotic medication, no main effects nor interactions were significant.

Other Correlates of CK Elevations

To examine the potential influence of other factors on these CK elevations, several comparisons were performed. CK levels of patients who were receiving neuroleptic medication at the time of the CK determination did not differ ($t = 1.42$, df = 161, n.s.) from levels of those who were not, nor did CK levels of patients who had received intramuscular injections (prior to CK determination) differ from those who had not ($t = 1.15$, df = 162, n.s.; see Table 4). The CK levels of patients with a principal clinical diagnosis

TABLE 3

Admission CK Level (Mean and SD) as a Function of Prior Restraints and Frequency, Severity, and Type of Subsequent Aggression: Main Effects and Interactions in Medicated vs. Unmedicated Patients

	Medicated Pts.			*Unmedicated Pts.*		
	F	*df*	*p*	*F*	*df*	*p*
Frequency of aggression	9.03	1,106	<.005	3.78	1,51	ns
Severity of aggression	26.58	1,106	<.001	2.18	1,51	ns
Type of aggression	15.37	1,106	<.001	2.78	1,51	ns
Prior restraints	14.65	1,106	<.001	2.70	1,51	ns
Frequency of aggression × Prior restraints	7.05	1,106	n.s.	4.21	1,51	ns
Severity of aggression × Prior restraints	10.50	1,106	<.005	1.48	1,51	ns
Type of aggression × Prior restraints	9.39	1,106	<.005	1.12	1,51	ns

TABLE 4

Relation of Clinical Variables to CK Elevations (U/l)

	Mean CK (SD)		*Analysis*	
Variable	*Present*	*Absent*	*t**	*p*
Neuroleptic medication	193.7 (375.2)	120.0 (100.3)	1.42	n.s.
Prior IM medication	243.8 (534.9)	158.1 (269.5)	1.15	n.s.
Prior restraints	473.4 (746.8)	123.7 (134.2)	5.14	<.001
Prior assault	437.9 (740.0)	137.4 (199.6)	3.91	<.001
Schizophrenia	165.9 (276.0)	173.7 (354.2)	1.60	n.s.
Major affective illness	236.6 (462.3)	155.6 (274.3)	1.24	n.s.

*df is 162 in all comparisons.

of schizophrenia (n = 85) did not differ from those of patients with other diagnoses (n = 77; t = 1.60, df = 160, n.s.). CK scores did not differ as a function of other clinical diagnoses, including major affective illness and alcohol abuse or dependence, diagnoses that have been linked to CK elevations (4). There were no documented incidents of patients with muscular hypertonicity nor patients involved in accidents. Only three patients had documented recent involvement in vigorous physical exercise (most patients came from prisons, jails, or other hospitals without access to a gymnasium). Their mean CK level (183 U/l), though slightly elevated, was not significantly different from the that of the other patients (168 U/l). There was no relationship (all p's > .10) between CK and age, daily average antipsychotic medication dose (expressed in chlorpromazine equivalents), agitation/hyperactivity (observed in 32 patients), nor EPS (observed in 13 patients).

Changes in CK Levels Over Time

A repeated-measures ANOVA of frequency of aggression scores over time (month 1 through month 6 of hospitalization) revealed no relationship between CK and changes in frequency of aggression over time. In patients (n = 66) who had multiple CK readings, t-tests showed no relationship between aggression scores and changes in CK levels over time. Patients with high mean CK (defined as mean CK > 200, n = 19) did not differ in aggression scores from patients with low mean CK. Patients with high variability of CK (defined as SD > 100, n = 21) did not differ in aggression scores from patients with low variability in CK.

Using CK in the Postdiction of Aggression

In order to examine the usefulness of CK in the postdiction of aggression, two models were compared. In the first model, patients with admission CK levels above 200 U/l were postdicted to engage in subsequent aggression. In the second model (one consistent with current practice), patients who engaged in assaultive behavior prior to CK determination were postdicted to engage in subsequent aggression. The sensitivity, specificity, positive predictive power, negative predictive power and overall di-

agnostic power of these two decision models demonstrate the greater effectiveness of the CK model (see Table 5).

DISCUSSION

Meltzer (3) proposed that the muscle isozyme (i.e., CK-MM) is released as a consequence of psychiatric illness. In a study of acutely psychotic patients by Vale *et al.* (21), this effect was described as a non-specific reaction to stress on the whole organism. More recently, Swartz and Breen (22) proposed that muscle enzyme release observed with psychiatric illness follows disruption of myocytes by hypoxia or energy depletion. They speculated that such a condition may follow physical agitation or extreme muscle tension of psychogenic origin.

TABLE 5

Sensitivity, Specificity, Positive Predictive Power (PPP), Negative Predictive Power (NPP) and Overall Diagnostic Power (DxP) of Two Postdictive Models of Aggression

	Postdiction of Aggression Based on	
	Prior Assaultiveness	*CK>200U/l*
Sensitivity	15%	100%
Specificity	94%	92%
PPP	65%	75%
NPP	59%	100%
DxP	60%	94%

Sensitivity is the proportion of true positives. Specificity is the proportion of true negatives. Positive predictive power is the probability that aggression occurred given the presence of the postdictor (i.e., prior assaultiveness or CK > 200 U/l). Negative predictive power is the probability that aggression did not occur given the absence of the postdictor. Overall diagnostic power is the proportion of correctly classified cases.

Stress-related elevations in plasma catecholamines levels have been related to myopathy in adults, children, and laboratory animals. Children treated with isoproterenol for severe asthma were observed to have elevated CK levels in conjunction with suspected myocardial injury (23). Adrenal tumors have also been associated with CK elevations and focal myositis in skeletal muscle without the presence of myocarditis (24). In a number of laboratory studies involving rats, dogs, hamsters, and rabbits, myocardial lesions and other myopathic abnormalities have been demonstrated with catecholamine administration (25). In the context of stress physiology, myopathic change may represent a stage along a continuum of stress-induced effects promoted by a long-term release of excess catecholamines. While some studies have failed to show an effect of stress on CK levels (26, 27), none have examined individuals with histories of both psychopathology and chronic aggression.

In the present study higher CK levels were consistently associated with higher patient aggression, as measured by three indices of aggression, namely frequency, severity, and type of aggression. The differences in CK levels were of statistical as well as clinical significance (i.e., all means were above 200 U/l), with the mean CK levels in the high aggression groups approximately twice that of the low aggression groups. These differences were not observed in patients who did not receive antipsychotic medication. Age, EPS, hyperactivity/agitation did not influence CK levels. No differences were noted among the various diagnostic groups.

Assaultiveness and use of restraints prior to CK determination, i.e., on admission, were associated with higher CK levels. However, even when these factors were included in the analyses, CK levels remained strongly associated with the subsequent aggression scores, and interactions were noted. The results of these ANOVAs may be summarized as follows: Higher subsequent aggression scores are associated with higher admission CK levels, prior assaultiveness and restraints are associated with higher CK levels, and the effect of prior assaultiveness and restraints on CK is much greater (the order of magnitude is approximately fivefold, i.e., mean CK > 600 U/l) among patients whose subsequent aggression is high than among those whose subsequent

aggression is low. This suggests that CK elevations may be a severity marker for recent assaultiveness. Among patients who are assaultive on admission and require restraints, CK elevations on admission differentiate those whose subsequent aggression is high from those whose subsequent aggression is low. It should be noted that this is only true in patients receiving neuroleptic medication. Fluctuations in subsequent CK levels during the course of hospital stay were not related to aggressive behavior.

The comparison of two postdictive models of aggression showed the clear superiority of the CK model that correctly classified 94% of cases as opposed to the model based on prior assaultiveness that only classified 60% of cases accurately. This comparison highlights the practical utility of using CK in predictive models of aggression. It should be noted that CK elevations were associated with increased aggression of any type, i.e., verbal as well as physical. In clinical situations, it is physical aggression (i.e., violence), that practitioners attempt to predict and prevent. CK was thus not solely postdictive of violence, but also of verbal aggression (i.e., insults, threats, etc.).

The present findings are consistent with the results of the study by Isaacs and Yesavage (9) in showing an association between assaultiveness and CK elevations. However, Isaacs and Yesavage did not find a link between admission CK levels and subsequent assaultiveness (i.e., while hospitalized). This discrepancy between the two studies may be attributable to the small number of patients in the Isaacs and Yesavage study. It is likely that their small sample resulted in insufficient power.

Admission CK levels appear to be a valuable potential predictor of aggressive behavior during hospitalization among patients receiving antipsychotic medications. Current factors used in predictions of violence include demographics, historical variables, symptomatic picture and prior aggression (28). The findings of this study add a new domain of variables (namely, biochemical) from which to gather information to predict violence. It is noteworthy that CK measurements may be obtained even from patients who are unwilling to discuss their histories in depth. Unlike other biochemical variables that have been linked to violence, such as serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) (29), CK is included in most laboratory

admission panels and thus readily available to the admitting clinician.

CK elevations are markers of muscular events and therefore should not be equated with actual violence or violence potential. As suggested above, this marker may be interpreted as a physiological response to a long-term pattern of aggressive behavior. The implications of the present study are that among violent individuals, significant CK elevations are suggestive of increased risk of violence. The present study suggests that CK elevations, as a single factor, may even have greater predictive utility than factors traditionally used to predict aggression (e.g., prior assaultiveness). It would not be warranted to make a prediction of violence in a patient simply based on a CK elevation, particularly since the present findings have not been replicated in a prospective study. Rather, along with factors such as age, sex, substance abuse, diagnosis, etc., the presence of a significant CK elevation should be included in the multifactorial conceptualization of a patient's risk for violence.

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Performance-Enhancing Substances: Is Your Adolescent Patient Using?

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Participation in athletic activities can benefit adolescents in numerous ways. In addition to keeping adolescents physically active, sports participation may enhance self-esteem, decrease stress, and encourage individuals to learn to work as a team; however, for some adolescents, the aim is not simply to enjoy sports participation but to win at all costs. They are not interested only in excelling as a team, but in striving to be the best as individuals as well. These adolescents may be at risk for using performance-enhancing substances to help them achieve their goals.

It is not only the high-level or "elite" athlete who may be attracted to these substances. For many, adolescence is a time of intense preoccupation with one's own body and body image. As puberty progresses, insecurities about a changing and growing body may lead an adolescent to consider using whatever means are available to attempt to alter his or her physical appearance. Coupled with the common perception among adolescents that they are unlikely to suffer negative consequences of their actions, this may lead adolescents to experiment with these substances.

The American Academy of Pediatrics defines a performance-enhancing substance as "any substance taken in nonpharmacologic doses specifically for the purposes of improving sports performance." This may include prescription medications, nutritional supplements, or illicit substances that individuals use to increase their strength, speed, or endurance or to control weight or alter body composition [1]. Several performance-enhancing substances are summarized in Table 1, along with selected positive and negative effects associated with their use. Virtually no research has been published on the effects or the risks of these substances in adolescents; therefore, this

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Table 1
Potential effects of selected performance-enhancing substances

Substance	Potential benefits	Potential risks and side effects
Anabolic-androgenic steroids	Increased strength Increased lean body mass Less muscle breakdown	Acne Hirsutism Gynecomastia Male pattern baldness Liver tumors Agitation/psychosis Virilization in girls Testicular atrophy Hypertension Premature closure of epiphyseal growth plates Infertility Ligamentous injury Precocious puberty in younger adolescents
Steroid hormone precursors (androstenedione, DHEA)	None proven	Increased estrogen levels in boys Possible androgenic effects, as above
Creatine	Increased strength Improved performance in short, anaerobic efforts Weight gain	Dehydration Muscle cramps Gastrointestinal symptoms Potential risk for renal toxicity
Human growth hormone	No proven effects on performance Decreases subcutaneous fat	Coarsening facial features Hypertension Cardiovascular disease Impaired glucose tolerance
Diuretics	Weight loss Enhanced muscle definition	Acute symptomatic dehydration Electrolyte imbalances
Nutritional supplements	None proven	Unregulated substances Potential side effects vary

Abbreviation: DHEA, dehydroepiandrosterone.

information is derived from adult studies. More extensive descriptions of the individual substances may be found elsewhere in this issue.

A large national survey of adolescent children of nurses revealed that 4.7% of boys and 1.6% of girls reported the weekly use of some kind of product to build muscles or improve the body's shape [2]. Substances used included protein powders and amino acid supplements, creatine, growth hormone, steroids, and steroid precursors. Five to 8% of adolescents stated that they have used creatine [3,4]. In a survey of high school football players in several Wisconsin schools, 30% reported that they used creatine [5]. Ongoing studies that assess substance use and other risky behaviors in nationally representative samples of high school students suggest that

approximately 3% of high school seniors have used anabolic-androgenic steroids [6,7]. Reported use is higher among boys than girls. Younger teens, including middle school and junior high school students, are nearly as likely as older adolescents to report having used steroids [8]. About 40% of high school seniors did not perceive a significant risk associated with the occasional use of anabolic steroids [7]; although it has not been studied, it is reasonable to hypothesize that younger teens are even less likely to understand the associated risks.

Therefore, those providing medical care for adolescents must be comfortable assessing adolescents for the use of performance-enhancing substances during sports physicals, at visits for sports-related injuries, and during routine health encounters. To be perceived as a credible source of information on this topic, providers must be knowledgeable about the specific substances in question and must acknowledge the potential benefits of using these substances in addition to the inherent risks. A "just say no" approach to counseling is unlikely to have the desired impact on an adolescent who is considering using these substances. By understanding the adolescent's goals and desired outcomes, it may be possible to help him or her achieve these outcomes by way of safer, more appropriate means.

Identifying the at-risk adolescent

Certainly, performance-enhancing substance use is most prevalent among athletes. The "typical" adolescent user of performance-enhancing substances is a male athlete who participates in a sport that demands high degrees of strength, power, size, or speed. He is more likely to use tobacco, alcohol, and other illicit substances as well [9,10]. The practice is more prevalent among football, baseball, wrestling, and gymnastics participants and those who engage in weight training and bodybuilding. Athletes who have reached a plateau in their training may turn to these substances to "break through" to a higher level of performance. Elite athletes, including those hoping for a college athletic scholarship or a career in professional sports, may use these substances to gain an "edge" over the competition. Young adolescents who already have specialized in a single sport year round, rather than engaging in a variety of athletic activities, may be on this pathway as well. Wrestlers and other athletes who need to meet certain weight requirements may use diuretics to achieve acute weight loss through dehydration; bodybuilders also may use such practices to achieve greater muscle definition before a competition.

Not every adolescent user of performance-enhancing substances fits this stereotype. More casual athletes, perhaps less informed about these substances, may believe that these substances are a quick fix or short cut to improved performance or muscle building. Those who are frustrated or unsatisfied with the results of the efforts they have made with their training may be tempted to try these products. Even nonathletes desiring an

improved physique may try using exogenous substances as a means of changing their bodies. Those who are particularly body conscious, who look at "muscle magazines" and related media, and who express an interest in becoming more muscular are at increased risk [2]. Adolescents who are being bullied may feel the need to "bulk up" to defend themselves or to intimidate others and may turn to these substances to help achieve that goal. Use is not confined to older adolescents; it is well documented among adolescents in middle school as well [8].

Adolescents are likely to do that which they perceive as normative. Therefore, knowing teammates, competitors, or other athletes who use these substances or believing that use is widespread may increase an individual's likelihood of using [8]. There may be a general feeling of acceptability of the practice among fellow athletes, rather than any stigma associated with use. Many athletes may believe that they are well educated about the substances that they are using (perhaps better educated than their health care providers), that they understand the risks and benefits, and that they are making appropriately informed decisions about using them. Those who believe that there is little risk for getting caught or reprimanded for the use of performance-enhancing substances may have less hesitation about trying them.

Adolescents with any of the above risk factors should be asked about their use of performance-enhancing substances. Those who are under pressure from their parents, teammates, or coaches to succeed in their athletic endeavors or whose identity and self-worth are largely defined by their sports participation and performance may be at particularly high risk. Recent documented changes in weight or body composition also should prompt the clinician to inquire about use.

Interviewing the adolescent

Before discussing this or any other sensitive topic with an adolescent, the provider should clarify his or her confidentiality policies with the patient and parent. It is reasonable to assure the patient that information regarding the use of performance-enhancing substances will be kept confidential unless that use is putting the patient's life or health in immediate danger. Adolescents have a right to know what their providers intend to do with information obtained in confidence, and they are more likely to answer questions honestly if they believe that their privacy will be protected [11,12].

Depth of interviewing necessarily depends on the amount of time that the provider has to spend with a patient. In brief encounters, such as during a visit for an acute sports-related injury, a single, directed screening question, "Are you using any substances to improve your sports performance?" may be used. In longer encounters, a more prolonged conversation about sports participation may naturally lead into a discussion of performance-enhancing substance use. This may include an assessment of hours per week spent training and playing sports, specific training practices, and how

important the adolescent feels it is to participate in and excel at his or her sport. The provider should inquire specifically about the adolescent's goals with regard to physique/body composition and sports performance, in the current season and in the long term (ie, are they anticipating a college or professional career in sports). Explore what, if anything, the adolescent sees as limitations or barriers to achieving these goals and what they have done or considered doing to try to overcome these obstacles.

A technique of "indirect questioning" may put an adolescent at ease when approaching sensitive topics. Using this technique, the provider begins by asking the adolescent his or her thoughts on a topic in general or as it applies to other people. Gradually, the questioning becomes more and more personal, ultimately culminating in a discussion of the adolescent's individual beliefs and experiences. For example, one may begin by asking adolescents how they feel about the practice of "doping" in professional sports and if they think that it is acceptable for younger, amateur athletes to use any or all of these substances as well. One may then ask if the adolescent is aware of any people their own age, perhaps even their own teammates, who engage in this practice. Finally, one can ask whether the adolescent has used any of these substances. This indirect technique gives the provider the opportunity to demonstrate an open, nonjudgmental consideration of this information, thereby making it more likely that the adolescent patient will feel comfortable disclosing his or her own beliefs and behaviors honestly.

When patients disclose that they have used substances to enhance their performance or physique, the provider must ask appropriate follow-up questions to clarify the extent of use and assess the degree of risk associated with these behaviors. This line of questioning should include what doses of substances the adolescent is using and how this dosing regimen was chosen. If a nutritional supplement is labeled for use at a specific dose, an adolescent may take a "more is better" approach and use even higher doses. Adolescents' use of the jargon common to the world of doping may be a clue that they are at particularly high risk for engaging in more dangerous patterns of use. These patients may report "cycling" (using a substance for a specific period of time and then discontinuing use for a time, perhaps when anticipating drug testing), "pyramiding" (using a regimen of increasing and decreasing dosing), or "stacking" (using multiple performance-enhancing substances simultaneously). Patients should be asked where they get information about these substances (eg, teammates, coaches, Internet, nutrition supplement shops). The provider should ask about the presence of side effects and if the adolescent uses any additional substances to minimize side effects or mask detection of the substances they use, should they be tested for drugs. Patients who admit to using injection drugs should be asked about needle-sharing practices. Because adolescents who use anabolic-androgenic steroids are more likely to engage in other risky behaviors, they should be asked about the use of tobacco, alcohol, and other illicit substances as well [13,14]. Specific questions that may be used when

interviewing an adolescent about performance-enhancing substance use, including indirect questions and follow-up questions, are listed in Box 1.

The physical examination

Most adolescents who use performance-enhancing substances have no distinguishing physical findings suggesting their use; however, a thorough physical examination may be useful in several regards. Height, weight, and body mass index should be obtained and compared with previous measurements. Body composition and degree of muscularity should be noted. Elevated blood pressure may suggest stimulant, human growth hormone, or anabolic-androgenic steroid use. In boys, anabolic-androgenic steroid use may lead to acne, male pattern baldness, gynecomastia, testicular atrophy, and severe striae. In addition to several of the above findings, girls may develop hirsutism, clitoral hypertrophy, and deepening of the voice. Injection drug users may have needle marks or evidence of skin abscesses. Creatine and other nutritional supplements are unlikely to have visible manifestations on physical examination.

Additional testing

Drug testing for exogenous substances may be performed, most commonly using gas chromatography with mass spectrometry; however, this testing may be of limited value. Availability of testing is limited, and some substances may not be detectable in a urine test. If urine testing is performed, the specimen ideally should be obtained under the direct observation of a same-gender member of the medical staff. If this is impossible, other measures to prevent dilution or contamination of the specimen should be considered, such as coloring the toilet water and disabling the sink in the restroom where the specimen is obtained. Although the threat or practice of routine drug screening may deter the "casual" user, a highly motivated athlete is likely to be one step ahead of the primary care provider with regard to techniques for hiding or masking his or her use. Furthermore, although such testing may limit the undesirable behavior, it is unlikely to lead the adolescent to give careful consideration to the issues of winning at all costs versus doing what he or she believes is morally right and justified. An open and honest discussion of the risks, benefits, and moral implications of doping may be more effective in this regard. A more extensive discussion of drug testing is contained elsewhere in this issue.

Patients who use injection drugs, particularly if they admit to any needle sharing, should be tested for hepatitis B, hepatitis C, and HIV. An electrocardiogram and metabolic profile, including glucose, a cholesterol and lipid panel, and liver function testing, may be considered in persons using human growth hormone and anabolic-androgenic steroids and their precursors. Electrolytes may be obtained in athletes who use diuretics or

Box 1. Questioning the adolescent patient about the use of performance-enhancing substances

Brief screening questions

- Are you using any substances to improve your sports performance?
- Are you using any substances to improve your body's appearance or strength?

Questions about sports participation and performance

- Are you satisfied with how well you do at your sport?
- What, if anything, do you think keeps you from being even better at your sport?
- Do you put a lot of pressure on yourself to improve your sports performance?
- Do other people in your life, like your parents, coaches, or teammates, put pressure on you to improve your performance?
- What are your short-term and long-term goals for sports participation (eg, plans to obtain an athletic scholarship)?

Questions about body image and satisfaction

- Are you happy with your current weight and body composition?
- Have you had any recent changes in your weight or body composition? How did you achieve this?
- Do you read magazines or visit Internet sites focused on bodybuilding or fitness?

Questions about performance enhancing substances

- How do you feel about professional athletes who engage in doping?
- Do you think it's okay for people your age to use substances to improve their sports performance? What kind of substances do you think it's okay to use?
- Do you think it's okay for schools to perform drug testing on their student athletes?
- Do you know any athletes at your school or on your team who use these substances? Which ones do they use?
- Has anyone ever encouraged you to use these substances?
- Have you ever used anything to improve your strength, physique, or performance?

Follow-up questions for positive screens

- Which substances have you tried? Which are you using currently?

- What dose do you use? Is this the recommended dose for this substance?
- Where do you get your information about using these substances?
- How did you determine the dosing regimen you use?
- Where do you get these substances?
- What changes have you seen in your body or performance since you started using this?
- Are you experiencing any side effects?
- Do you use any additional substances to help lessen these side effects or to avoid having a positive drug screen?
- Where do you get your needles? Do you share needles (if applicable)?
- Do you understand that it is illegal to use certain substances without a doctor's prescription?
- Do you use tobacco, alcohol, or any other drugs?

excessive quantities of creatine. Keep in mind that these laboratory values are normal in most athletes who use performance-enhancing substances; normal values should not provide reassurance that the adolescent is not using.

Prevention

Perhaps the most important role of the primary care provider is to discuss performance-enhancing substance use with adolescents before they consider or initiate use. Athletes should be encouraged to consider how they feel about competition, the importance of winning, and the importance of achieving their personal best performance. What constitutes fair versus unfair competition? How do performance-enhancing substances fit into this model of fair competition? Does the adolescent make a distinction between "legal" and banned substances in this regard? Patients who voice opposition to the use of these substances should be supported and their decisions reinforced. Skills to resist peer pressure to consider using should be taught, perhaps by way of role playing.

A categorical "just say no" approach to counseling is unlikely to be effective and may be detrimental. Recent media attention to doping in professional sports has contributed to widespread acceptance that the use of some substances does confer an advantage to the user. Some patients already may have seen beneficial effects in themselves or peers who are users. To be seen by the athlete as a credible source of health information, a provider must acknowledge that some substances, particularly

anabolic-androgenic steroids, clearly have been shown to increase muscle mass and potentially improve performance. Creatine also has been associated with improved performance in some studies. The provider may proceed, however, by providing additional information about which some adolescents may not be aware. These two substances are only effective when used in conjunction with intense resistance training; they do not provide an effortless "short cut" to bigger muscles. Furthermore, they offer only a small benefit over a healthy diet and a rigorous training regimen. For the elite athlete, this may be the tiny edge that one needs over the competition, but the typical middle school or high school athlete may not perceive a significant difference in one's performance. The provider may point out that simple measures, such as getting enough sleep, maintaining adequate hydration, and quitting smoking, may have a greater positive impact on sports performance than using supplements. When discussing creatine use, adolescents need to be aware that approximately 20% to 30% of the population seem to be "nonresponders" who will not see beneficial effects [15]. Furthermore, the American College of Sports Medicine, while acknowledging the positive effects of creatine, does not recommend its use in persons younger than 18 years of age [16]. It is speculated, but not proven, that creatine may serve as a "gateway" drug, the use of which may lead to the use of more dangerous performance-enhancing substances.

Adolescents considering the use of anabolic-androgenic steroids need to know the potential medical risks and side effects associated with the use of these substances, including acne, striae, psychiatric symptoms, the development of liver tumors, and adverse cardiovascular effects. Female users may develop hirsutism, deepening of the voice, male pattern baldness, clitoral enlargement, and menstrual irregularities. They need to understand that some of the virilizing features may be irreversible. Boys may experience gynecomastia, hair loss, and testicular atrophy. Although the cosmetic effects may not be the most concerning risks from the provider's perspective, these concrete images, such as irreversible gynecomastia for a boy or hirsutism for a girl, may have a greater psychologic impact than more dangerous but more abstract risks, such as liver disease. Adolescents who are considering using steroids or other injection drugs should be educated regarding the infectious risks associated with sharing needles.

Many advertisements for performing-enhancing nutritional supplements are aimed specifically at the adolescent and young adult demographic. Some adolescents are becoming more media savvy and may respond well to the idea that those running the multimillion dollar industry of nutritional supplements are counting on young people to be vulnerable to their marketing claims. Remind them that these substances are not regulated by the US Food and Drug Administration in the same manner as prescription medications and that the claims made by advertisers often are not substantiated. Furthermore, quality control during the processing and packaging of these products is variable, and there is no guarantee that the contents of the

container are reflected accurately on the label. Some products that are marketed as legal nutritional supplements conceivably may contain substances that are banned or dangerous to enhance their perceived efficacy. This could result in a positive drug screen or other adverse medical effects in an athlete who believes that he or she is using a safe substance. Of course, this also makes it impossible for the provider to counsel on the safety of these products with any degree of certainty.

Counseling a patient not to take a particular course of action (eg, not to use performance-enhancing substances) is likely to be more effective if the provider can offer the patient alternative means of achieving the desired outcomes. Therefore, a broader discussion of an adolescent's motivation for considering the use of these substances is warranted. Ask the adolescent to describe his or her performance goals or goals for physical appearance. Help the adolescent to explore alternative ways to achieve these goals. Point out some of the adolescent's modifiable lifestyle choices that may be impairing performance, such as smoking cigarettes or marijuana, using alcohol, poor sleep habits, or poor conditioning. Become knowledgeable about local referral resources that may assist patients in making appropriate changes. Most adolescents have room for improvement in their diets; a sports nutritionist can help them to make better food choices to optimize their health and performance. The American College of Sports Medicine endorses the safety of strength training, even in preteenagers [17]; a qualified trainer can help them develop a safe and effective training regimen. Sensible weight control programs, such as Weight Watchers, are widely available in many communities and are appropriate for adolescents who wish to lose weight in a healthful manner. For adolescents with symptoms of a mood or anxiety disorder or who are experiencing stressful psychosocial situations, getting help from a mental health provider may enhance their overall well-being, which may be reflected by better performance in sports and other extracurricular activities.

Lastly, parents of adolescent athletes should be educated as well. Some may not appreciate the potential dangers of using certain performance-enhancing substances; others may know that they can be harmful but may not realize how widespread their use is, even at the middle school level. Parents should know that the American Academy of Pediatrics strongly condemns the use of performance-enhancing substances [1]. They should be encouraged to have ongoing discussions with their children, particularly those who are athletes, about this issue. These discussions become even more important as athletes aspire to higher and higher levels of performance in their sport. Parents should make their own views on the subject clear and communicate their expectations explicitly and repeatedly to their children. They should remind their children of all of the positive aspects of sports participation and encourage them to focus on these aspects and not solely on winning. They should attempt not to let their child's self-esteem be tied too closely to winning or performance and should encourage them instead

to take pride in being fair and honest in their approach to sports participation. Parents should be aware of the side effects of dangerous substances and what to look for in their own child.

Summary

Adolescents have variable knowledge about performance-enhancing substances, but many athletes and nonathletes may be considering their use. Young adolescents are as likely as older adolescents to use these substances. Although most nutritional supplements are not likely to cause serious harm, they are largely unregulated and their safety cannot be assured. Anabolic-androgenic steroids, although illegal, are readily available to athletes who seek them out. Although the potential adverse effects of steroid use are unequivocal and should be emphasized, their positive effects must be acknowledged as well if a provider is to have a meaningful discussion with an athlete regarding their use. While discouraging the use of performance-enhancing substances, providers should be prepared to assist their patients in achieving their performance and appearance goals in alternative, healthful ways.

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See corresponding editorial on page 11.

Food sources of nitrates and nitrites: the physiologic context for potential health benefits[1–3]

Norman G Hord, Yaoping Tang, and Nathan S Bryan

ABSTRACT

The presence of nitrates and nitrites in food is associated with an increased risk of gastrointestinal cancer and, in infants, methemoglobinemia. Despite the physiologic roles for nitrate and nitrite in vascular and immune function, consideration of food sources of nitrates and nitrites as healthful dietary components has received little attention. Approximately 80% of dietary nitrates are derived from vegetable consumption; sources of nitrites include vegetables, fruit, and processed meats. Nitrites are produced endogenously through the oxidation of nitric oxide and through a reduction of nitrate by commensal bacteria in the mouth and gastrointestinal tract. As such, the dietary provision of nitrates and nitrites from vegetables and fruit may contribute to the blood pressure–lowering effects of the Dietary Approaches to Stop Hypertension (DASH) diet. We quantified nitrate and nitrite concentrations by HPLC in a convenience sample of foods. Incorporating these values into 2 hypothetical dietary patterns that emphasize high-nitrate or low-nitrate vegetable and fruit choices based on the DASH diet, we found that nitrate concentrations in these 2 patterns vary from 174 to 1222 mg. The hypothetical high-nitrate DASH diet pattern exceeds the World Health Organization's Acceptable Daily Intake for nitrate by 550% for a 60-kg adult. These data call into question the rationale for recommendations to limit nitrate and nitrite consumption from plant foods; a comprehensive reevaluation of the health effects of food sources of nitrates and nitrites is appropriate. The strength of the evidence linking the consumption of nitrate- and nitrite-containing plant foods to beneficial health effects supports the consideration of these compounds as nutrients. *Am J Clin Nutr* 2009;90:1–10.

INTRODUCTION

The health effects of the dietary consumption of vegetables and fruit have been attributed to their constituents, including vitamins, minerals, fiber, and so-called nonnutritive substances such as flavonoids and glucosinolates to name a few (1–3). Dietary supplements containing food components such as β-carotene and antioxidant vitamins such as vitamin A and E have been used in secondary prevention trials for the prevention of lung cancer (4, 5). These trials found that β-carotene, alone or in combination with vitamin E or retinyl palmitate, increased the incidence of lung cancers and cardiovascular disease mortality rates (6). Indeed, meta-analyses of primary and secondary cancer prevention trials of dietary antioxidant supplements consistently show a lack of efficacy and an increased risk of mortality (7). Clearly, more research is needed to identify the nutrients and food components of vegetables and fruit associated with a decreased risk of cardiovascular disease and cancer.

Whereas the health benefits of vegetables and fruit may derive from the contribution of their constituents to food patterns such as the Mediterranean-type pattern (8–10), recent research has found specific foods to be associated with a decreased risk of cardiovascular disease. Recent prospective epidemiologic studies have shown that green leafy vegetables are among the foods most protective against coronary heart disease and ischemic stroke risk (11, 12). The Dietary Approaches to Stop Hypertension (DASH) studies found that diets rich in vegetables (ie, 8–10 servings) and low-fat dairy products can lower blood pressure to an extent similar to that achieved with single hypotensive medications (13, 14). The blood pressure–lowering effect of this diet was hypothesized to be attributable to the high calcium, potassium, polyphenols, and fiber contents and low sodium and animal protein contents (15). These and other findings point to a less widely acknowledged but biologically plausible hypothesis: the content of inorganic nitrate (NO_3^-) in certain vegetables and fruit can provide a physiologic substrate for reduction to nitrite (NO_2^-), nitric oxide, and other metabolic products (NO_x) that produce vasodilation, decrease blood pressure, and support cardiovascular function (16–18). Interestingly, both potassium nitrite, in 1880, and potassium nitrate, in 8th century China, were known to mediate hypotensive and antianginal actions,

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respectively (19, 20). The goals of this review are to *1*) provide a physiologic context for the potential health benefits of dietary nitrite and nitrate from plant foods, and *2*) support a growing consensus for a comprehensive reevaluation of the health benefits and risks associated with dietary sources of nitrates and nitrites.

NITRITE AND NITRIC OXIDE PRODUCTION IN THE VASCULATURE AND IN TISSUES: 2 SYSTEMS, REDUNDANT FUNCTIONS

New discoveries in the field of nitrate and nitrite biology have provided mechanistic insights into the potential new physiologic roles of dietary nitrate and nitrite and their potential health benefits. A brief introduction to the biology of nitric oxide production in the vasculature and nitric oxide–requiring tissues will provide the appropriate context for understanding the importance of dietary nitrate and nitrite. There is a consensus that dietary nitrates are essentially inert and acquire biological activity only after reduction to nitrite. As such, nitrate serves as a source, via successive reduction, for the production of nitrite and nitric oxide as well as other metabolic products. The late Speaker of the US House of Representatives, Representative Thomas "Tip" O'Neil, famously stated "All politics is local." There is no more apropos analogy in biology than the regulation of the availability of nitrogen oxides—by localization, oxygen tension, pH, inflammatory microenvironment, and organ and tissue specificity—that determines how much nitrate, nitrite, nitric oxide, and other NO_x species to which tissues will be exposed.

VASCULAR NITRIC OXIDE PRODUCTION

Normal functioning of human vasculature requires both the presence of nitrite and nitric oxide along with the necessity to respond to these important signaling molecules (21, 22). The generation of up to ≈70% of systemic nitric oxide is accomplished by endothelial nitric oxide synthase (eNOS), one of 3 members of the NOS family of enzymes, in the vascular endothelium (23). These enzymes synthesize nitric oxide from the amino acid L-arginine and molecular oxygen to accomplish vasodilation, blood pressure regulation, inhibition of endothelial inflammatory cell recruitment, and platelet aggregation (21). As a result, the normal production of nitric oxide and nitrite and the ability of the endothelium to respond to these species may prevent various types of cardiovascular disease, including hypertension, atherosclerosis, and stroke (24).

The biological effects of nitric oxide are caused by the initiation of cyclic GMP (cGMP)–mediated intracellular signals in the vascular wall. Two other members of the NOS family have neuronal functions (nNOS) and inflammatory immune functions (inducible NOS or iNOS) (25). In neuronal tissue, nNOS provides nitric oxide for normal neuron function. The function of iNOS is an essential signaling mechanism in the innate immune response (26). In tissues experiencing chronic inflammation, such as inflamed bowel tissue in ulcerative colitis, iNOS can generate high concentrations of nitric oxide that promote carcinogenesis by inhibiting apoptosis, enhance prostaglandin formation, and promote angiogenesis in the early stage of carcinogenesis (27–29). In atherosclerosis, hypoxic conditions combined with an oxidative environment can limit eNOS-derived nitric oxide production; nitrite can directly induce vasodilation in hypoxic endothelium (30). Indeed, the Bryan laboratory has shown that nitrite can restore vascular tone after ischemia/reperfusion and substitutes for loss of eNOS-derived nitric oxide in eNOS-deficient mice (31, 32).

Unlike the provision of eNOS-derived nitric oxide to the endothelium to maintain vasomotor tone, nitric oxide production from nitrite occurs primarily in tissues (33). There are 2 systems of reducing nitrate to nitrite in mammals. The first system identified to accomplish this was the action of commensal gram-negative bacteria on the tongue to reduce salivary nitrate (34). Concentrations of plasma nitrate in the saliva occur as part of enterosalivary circulation of dietary nitrate (35). Approximately 25% of ingested nitrate is secreted in saliva, where some 20% (or ≈5–8% of the nitrate intake) is converted to nitrite by commensal bacteria on the tongue (36). These anaerobic bacteria on the dorsal surface of the tongue use nitrate as an alternative electron acceptor to produce energy. Indeed, use of an antibacterial mouthwash after consumption of dietary nitrate (10 mg/kg in water) attenuates the expected postprandial rise in plasma nitrite (37). In the proximal small intestine, nitrate is rapidly absorbed with high bioavailability (100%) (38). The nitrite supplied to the gastrointestinal tract serves to enhance gastric mucin production (39) and can serve as a substrate for generation of nitrogen oxides for antimicrobial actions and support of gastric homeostasis (40).

NITRIC OXIDE PRODUCTION IN TISSUES

Recently, nitric oxide synthesis in healthy tissues has been shown to occur independently of the L-arginine–NOS pathway (41); dietary provision of nitrates and nitrites may account for approximately half of steady state nitric oxide concentrations. Because inorganic nitrate is considered a biologically inert compound, the reduction of nitrate to nitrite is necessary for nitrite to serve as a substrate for nitric oxide production. The Lundberg group at the Karolinska Institute has shown, for the first time, that mammalian enzymes have nitrate reductase activity—a function previously thought to be carried out only by bacterial nitrate reductases (41). As such, several different mammalian enzymes and metalloproteins have been shown to possess nitrate reductase activity, including xanthine oxidoreductase (XOR), aldehyde oxidase (AO), heme proteins, and mitochondria (41, 42). Nitric oxide synthesis in tissues, therefore, can occur through a reduction of nitrate to nitrite and nitrite can be subsequently reduced to nitric oxide. Nitrite reduction to nitric oxide can be carried out by numerous metalloproteins, enzymes, and compounds with redox potential, including hemoglobin (43), deoxyhemoglobin, deoxymyoglobin, XOR, vitamin C, and polyphenols (41). As noted above, nitrite reduction to nitric oxide is greatly enhanced during the stress of hypoxemia and ischemia (44). These redundant physiologic systems for the provision of nitric oxide under normoxic or hypoxic conditions indicate that nitrite may serve as systemic reservoir for nitric oxide production.

Emerging evidence from animal models and human clinical studies indicates that nitrite exerts unique intracellular signaling properties that mediate physiologic functions independent of its role as a source of nitric oxide in tissues by reduction (24). Nitrite

infusion in humans induces rapid local vasodilation, reduces blood pressure acutely, serves as an endocrine reservoir of nitric oxide, and, unlike organic nitrates, does not induce tolerance (45, 46). Nitrite has also been shown to play a role in mitochondrial respiration (47), cardiac function (48), activation the α form of the estrogen receptor (49), and exertion of antiapoptotic effects (50). Because nitrite is a biologically active compound resulting from nitrate reduction in tissues, significant physiologic benefits may be associated with the provision of nitrite from dietary sources.

REGULATION OF THE NITRITE ECONOMY: ROLE OF DIET, TISSUE NITRATE REDUCTASES, AND DISEASE STATES

The stepwise reduction of nitrate to nitrite to nitric oxide is, by necessity, an inefficient process by which each step yields a 3-log lower concentration of product than substrate (41). Therefore, a 10 mg/kg infusion of nitrate given over 5 min yielded a plasma concentration of nitrite of $\approx$1 μmol/L and resulted in ostensibly nitric oxide–mediated vasodilation after experimentally induced ischemia (41). Typical plasma concentrations, half-lives, and sources of nitrate, nitrite, and nitric oxide are shown in **Table 1** (45, 51). The 1- to 5-min half-life of nitrite is intermediate between that of nitrate (5–8 h) and nitric oxide (milliseconds) (44). Notably, the short half-life of nitric oxide results from efficient oxidation of nitric oxide to nitrite and other nitrogen oxides, such as *N*-nitroso compounds by enzymes (so-called nitric oxide oxidases) that use transition metals in their active sites, such as copper-containing ceruloplasmin (52), myeloperoxidase (which uses heme iron as a cofactor), and even endothelial NOS (53). Oxidation of nitric oxide to nitrite and nitrite to nitrate contributes to the pool of NO_x compounds that serve as signaling molecules systemically or as a local substrate for nitric oxide production. In situations such as iNOS-mediated inflammatory processes in ulcerative colitis, the large concentrations of nitric oxide produced can lead to high concentrations of more stable nitric oxide oxidation products such as nitrite and nitrate. The elegant physiologically redundant mechanisms by which nitrite and nitrate are produced and reformed by oxidation of nitric oxide to ensure an abundant supply for the myriad processes that require them for adequate functioning are illustrated in **Figure 1**. Dietary sources of nitrate and nitrite may bolster the reserve of these compounds for optimal functioning through periods of physiologic stress and diseases characterized by endothelial dysfunction (31, 32).

TABLE 1
Plasma concentrations, half-lives, and sources of NO_x species (nitrate, nitrite, and nitric oxide)[1]

NO_x species	Fasting plasma	Half-life	Exogenous or endogenous source
	nmol/L		
Nitrate	20–50,000	5–8 h	Diet or endogenous oxidation of nitrite
Nitrite	100–500	1–5 min	Endogenous nitrate, diet, oxidation of nitrite
Nitric oxide	<1	1–2 ms	Endogenous nitrite

[1] Data from references 44 and 51.

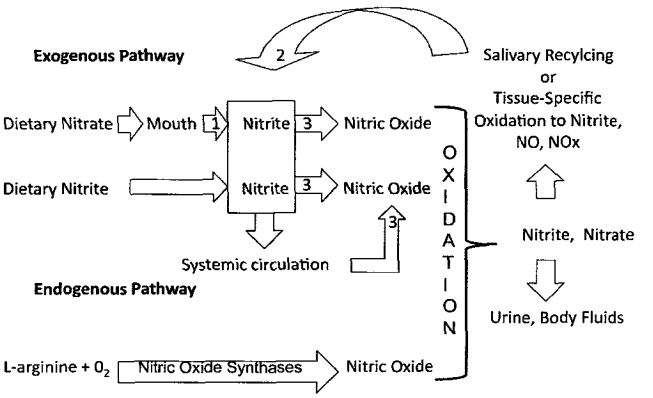

FIGURE 1. A schematic diagram of the physiologic disposition of nitrate, nitrite, and nitric oxide from exogenous (dietary) and endogenous sources. The action of bacterial nitrate reductases on the tongue and mammalian enzymes that have nitrate reductase activity in tissues are noted by the number 1. Bacterial nitrate reductases are noted by the number 2. Mammalian enzymes with nitrite reductase activity are noted by the number 3.

SOURCES OF ENDOGENOUS AND DIET-DERIVED NITRIC OXIDE GENERATION

In addition to the provision of nitrate and nitrite by diet or via the oxidation of nitric oxide to nitrite, vascular and gastrointestinal nitric oxide production can be enhanced through various means based on lifestyle and food choices. Physical activity, commensal bacteria, and dietary factors can influence nitric oxide production. Exercise enhances nitric oxide production in vascular endothelium (54) and postexercise plasma nitrite concentrations have been proposed as an index of exercise capacity (55). In fact, aging is associated with an impaired capacity of the vasculature to increase plasma nitrite during exercise (56). Strikingly, it has been found that dietary nitrate supplementation, at concentrations achievable by vegetable consumption, results in more efficient energy production without increasing lactate concentrations during submaximal exercise (57).

Foods can increase the generation of nitric oxide in the gastrointestinal tract via the polyphenolic content of, for example, apples or red wine (58, 59). Pomegranate juice has been shown to protect nitric oxide from oxidation while enhancing its biological activity (60). The metabolic activity of commensal bacteria in the gastrointestinal tract and probiotic bacteria also provide nitric oxide from nitrite, and to a lesser extent, from nitrate (61, 62). Whereas data estimating the contribution of the microbiota, including probiotic bacteria, to the generation of nitric oxide are speculative, they raise the possibility that the gastrointestinal production of nitric oxide and NO_x is biologically plausible. These data add layers of complexity to the estimation of nitrate/nitrite exposure levels in vivo and the determination of whether specific foods or lifestyle choices can significantly affect the production and metabolic disposition of dietary and endogenous NO_x species.

QUANTIFYING THE NITRITE ECONOMY

Given the complex interactions between nitrite and nitrate of dietary origin, the endogenous production of nitrate and nitrite from nitric oxide and other nitrogen oxides (NO_x) (Figure 1), the effect of physiologic conditions such as atherosclerosis and inflammatory disease, dietary sources of NO_x, and physical

activity, nitrate, and nitrite balance studies are not, at present, feasible. Therefore, a simple characterization of an optimal concentration of dietary nitrate and nitrite based on an overall picture of the nitrate and nitrite economy is not possible. However, we can make 2 generalizations that summarize our current knowledge. First, most nitrite utilization and nitric oxide production occur in healthy individuals in 2 compartments: vascular and somatic tissues. Normal vascular function requires nitric oxide production from the L-arginine-NOS pathway; in ischemic conditions, nitrite can substitute for L-arginine-NOS-derived nitric oxide (31, 32). Most healthy somatic tissues possess mammalian enzymes that exert nitrate reductase activity (the tongue utilizes nitrate reductases of commensal bacteria) to generate biologically active nitrite to maintain gastrointestinal and cardiovascular health. In inflammatory conditions, iNOS in epithelial and immune cells can produce nitric oxide as part of the innate immune response. The second generalization is that in cardiovascular disease states characterized by hypoxia and/or ischemia/reperfusion injury, eNOS-supplied nitric oxide may be limiting and nitrite may be used to support vascular function under these conditions. However, concern has been expressed that nitrite may be reduced to nitric oxide under normoxic conditions (63) and that, under these conditions, nitrate and nitrite may inhibit steroidogenesis in vitro and in vivo (64).

The data supporting the in vivo conversion of nitrates and nitrites to nitric oxide has implications for dietary consumption of foods high in nitrate and nitrite. As such, nitrate- and nitrite-containing foods may supply nitrite in situations in which substrates for endogenous NO_x production are limiting, as in cardiovascular conditions, to support cardiovascular and gastrointestinal function. As such, when the dietary intake of nitrate and nitrite is low and there is no additional endogenous sources of NO_x (eg, gastrointestinal infections involving iNOS activation), the endogenous production of nitrate, via oxidation of nitric oxide and nitrite, provides more substrate for nitric oxide production than dietary sources. Long-term consumption of diets containing high levels of nitrate and nitrite may have important implications for providing health benefits by ensuring high concentrations of nitrogen oxides as a "reserve" for tissue defense and homeostasis in stress and disease.

DIETARY SOURCES OF NITRATE AND NITRITE

Dietary nitrate intake is determined by the type of vegetable consumed, the levels of nitrate in the vegetables (including the nitrate content of fertilizer), the amount of vegetables consumed, and the level of nitrate in the water supply (65). As such, the nitrate content of organic vegetables may be less than that of vegetables grown in the presence of nitrogen-containing fertilizers. The primary determinants of nitrite consumption are the levels of nitrites in cured, processed meats and the consumption level of these products. A recent survey of vegetable nitrate concentrations in the European Union states and Norway based on ≈42,000 submitted analytic results showed a variation ranging from a low of 0.1 mg/100 g (peas and Brussels sprouts) to a high of 480 mg/100 g (rucola or rocket) (66). The nitrate and nitrite contents of edible vegetable components are listed in **Table 2** (from reference 67). A list of vegetable varieties grouped in ascending order of nitrate content are shown in **Table 3** (from reference 68). In terms of plant anatomy, the nitrate content of vegetable organs can be listed in descending order (most to least) as petiole > leaf > stem > root > influorescence > tuber > bulb > fruit > seed (69). The accumulation of nitrate is subject to factors such as genotype, soil conditions, growth conditions (ie, nitrate uptake, nitrate reductase activity, and growth rate), and storage and transport conditions (65, 70). For example, the average nitrate content of spinach collected from 3 different markets in Dehli, India, varied from 71 to 429.3 mg/100 g fresh weight (FW) (70). These data dictate that caution be observed in linking the biological effects of leafy vegetables (and other nitrate-containing vegetables and fruit) to specific health effects, particularly in observational epidemiologic studies.

DIETARY NITRATE AND NITRITE INTAKE ESTIMATES

The mean intake estimates for nitrate and nitrite in the United States and Europe vary by investigator but are consistent and comparable. International estimates of nitrate intakes from food are 31–185 mg/d in Europe and ≈40–100 mg/d in the United States (71, 72). The bioavailability of dietary nitrate is 100% (38). Nitrite intakes vary from 0 to 20 mg/d (65). Nitrate intakes from sources other than vegetables, including drinking water and cured meats, has been estimated to average 35–44 mg/person per day for a 60-kg human (66). On the basis of a conservative recommendation to consume 400 g of different fruits and vegetables per day at median nitrate concentrations, the dietary concentration of nitrate would be ≈157 mg/d (66). In the European Union, where fruit consumption (average nitrate concentration: <10 mg/kg FW) constitutes more than half of the recommended intake of 400 g, actual nitrate intakes would be ≈81–106 mg/d before additional nitrate losses from washing, peeling, and/or cooking are taken into consideration.

A CASE STUDY IN NITRATE AND NITRITE INTAKE ESTIMATES BASED ON A CONVENIENCE SAMPLE

Due to the variability in nitrate and nitrite concentrations of foods reported in Tables 2 and 3, we conducted nitrate and nitrite

TABLE 2
Nitrate and nitrite contents of edible components of vegetables[1]

Vegetable types and varieties	Nitrite	Nitrate
	mg/100 g fresh weight	*mg/100 g fresh weight*
Root vegetables		
Carrot	0.002–0.023	92–195
Mustard leaf	0.012–0.064	70–95
Green vegetables		
Lettuce	0.008–0.215	12.3–267.8
Spinach	0–0.073	23.9–387.2
Cabbage		
Chinese cabbage	0–0.065	42.9–161.0
Bok choy	0.009–0.242	102.3–309.8
Cabbage	0–0.041	25.9–125.0
Cole	0.364–0.535	76.6–136.5
Melon		
Wax gourd	0.001–0.006	35.8–68.0
Cucumber	0–0.011	1.2–14.3
Nightshade		
Eggplant	0.007–0.049	25.0–42.4

[1] Data from reference 67.

TABLE 3
Classification of vegetables according to nitrate content[1]

Nitrate content (mg/100 g fresh weight)	Vegetable varieties
Very low, <20	Artichoke, asparagus, broad bean, eggplant, garlic, onion, green bean, mushroom, pea, pepper, potato, summer squash, sweet potato, tomato, watermelon
Low, 20 to <50	Broccoli, carrot, cauliflower, cucumber, pumpkin, chicory
Middle, 50 to <100	Cabbage, dill, turnip, savoy cabbage
High, 100 to <250	Celeriac, Chinese cabbage, endive, fennel, kohlrabi, leek, parsley
Very high, >250	Celery, cress, chervil, lettuce, red beetroot, spinach, rocket (rucola)

[1] Data from reference 68.

analyses on a convenience sample of vegetables, a commercial vegetable juice beverage (V8; Campbell Soup Co, Camden, NJ), fruit, fruit juices, as well as fresh and processed meats (**Tables 4** and **5**). Vegetables with the highest nitrate concentrations in our sample included spinach (740 mg/100 g FW), collard greens (320 mg/100 g FW), mustard greens (120 mg/100 g), broccoli (40 mg/100 g FW), and tomato (39 mg/100 g FW). Banana, apple sauce, and oranges had nitrate and nitrite concentrations (mg/100 g FW) of 5 and 0.009, 0.3 and 0.008, and 0.8 and 0.015, respectively. Vegetable and fruit juices had nitrate and nitrite concentrations (mg/L FW) of 27.6 and 0.04 (carrot juice), 26.1 and 0.09 (V8 juice), 12.9 and 0.07 (pomegranate juice), 9.1 and 0.14 (cranberry juice), and 0.6 and 0.01 (acai juice). Note that a desiccated vegetable dietary supplement (Nature's Way Garden Veggies; Nature's Way Products Inc, Springville, UT) had the highest nitrate and nitrite concentrations of any food tested, ie, 27,890 and 10.5 mg/100 g FW, respectively. Each capsule of this supplement contains 900 mg desiccated vegetable product, and label recommendations suggest a daily intake of 2 capsules daily, which equates to >500 mg nitrate and 0.2 mg nitrite per day. Hot dogs, ham, pork tenderloin, bacon, and nitrate- or nitrite-free bacon had nitrate and nitrite concentrations (mg/100 g FW) of 9 and 0.05, 0.9 and 0.89, 3 and 0, 6 and 0.38, and 3 and 0.68, respectively.

MODELING NITRATE AND NITRITE INTAKES BASED ON THE VEGETABLE AND FRUIT CONTENT IN THE DASH DIET PATTERN

Our data, considered together with data in Tables 2 and 3, make it plain that, because of the wide variation in nitrate and nitrite contents of vegetables, fruit, and their juices, practicing the oft-quoted dietary recommendation "Eat your fruits and vegetables"

TABLE 4
Mean nitrate and nitrite contents of a convenience sample of juices

Juices	Nitrate	Nitrite
	mg/L; ppm	*mg/L; ppm*
Acai	0.56	0.013
Carrot	27.55	0.036
Cranberry	9.12	0.145
Green tea	0.23	0.007
Pomegranate	12.93	0.069
Vegetable juice[1]	26.17	0.092

[1] V8; Campbell Soup Co (Camden, NJ).

TABLE 5
Mean nitrate and nitrite contents of a convenience sample of fruit, vegetables, meats, and processed meats[1]

	Nitrates	Nitrites
	mg/100 g	*mg/100 g*
Fruit		
Apple sauce	0.3	0.008
Banana	4.5	0.009
Fruit mix	0.9	0.08
Orange	0.8	0.02
Vegetables		
Broccoli	39.5	0.07
Carrots	0.1	0.006
Cole slaw	55.9	0.07
French fries	2.0	0.17
Ketchup	0.10	0.13
Mustard greens	116.0	0.003
Salad mix	82.1	0.13
Spinach	741	0.02
Tomato	39.2	0.03
Vegetable soup	20.9	0.001
Desiccated vegetable dietary supplement[2]	27,890	10.5
Meats/processed meats		
Bacon	5.5	0.38
Bacon, nitrite-free	3.0	0.68
Ham	0.90	0.89
Hot dog	9.0	0.05
Pork tenderloin	3.3	0

[1] Nitrate and nitrite concentrations were quantified by ion chromatography (ENO 20 Analyzer; Eicom, Kyoto, Japan). Analysis of foods reflects the mean value from triplicate or quadruplicate analyses.

[2] Nature's Way Garden Veggies (1 capsule; 900 mg desiccated vegetables; Nature's Way Products Inc, Springville, UT).

may not translate into high nitrate and nitrite concentrations in the diet. We set out to model this variation by using the vegetable and fruit components of the DASH dietary pattern (73) that involved choosing particular foods with a high or low nitrate content. Two hypothetical vegetable and fruit consumption patterns based on the DASH diet (1 cup raw leafy vegetables, 1/2 cup cut-up raw or cooked vegetables, 1/2 cup vegetable juice, 1 medium fruit, 1/4 cup dried fruit, 1/2 cup fruit juice, or 1/2 cup fresh, frozen, or canned fruit), which contains foods that are low or high in nitrate, are shown in **Table 6**. The high-nitrate DASH diet would result in the consumption of 1222 mg nitrate and 0.351 mg nitrite compared with the low-nitrate DASH diet that yields 174 mg nitrate and 0.41 mg nitrite. These analyses make evident that consuming a dietary pattern such as the DASH diet can yield differences in nitrate intake that vary by ≈700%.

POTENTIAL HEALTH RISKS OF EXCESSIVE NITRATE AND NITRITE EXPOSURE

Analogous to all essential or indispensable nutrients, intake of excess nitrate and nitrite exposure is, in specific contexts, associated with an increased risk of negative health outcomes. A set of Dietary Reference Intake (DRI) categories are set by the Food and Nutrition Board of the National Academy of Sciences for essential nutrients to clearly define, where possible, the contexts in which intakes are deficient, safe, or potentially excessive. These DRI categories include the Recommended Dietary Allowance (RDA), Adequate Intake (AI), Tolerable Upper Level Intake (TUL), and Estimated Average Intake (EAI) (74). The

TABLE 6
Hypothetical dietary nitrate and nitrite intakes based on food and juice serving recommendations for vegetables and fruit based on the Dietary Approaches to Stop Hypertension (DASH) dietary pattern[1]

Food pattern and serving size	Nitrate content	Nitrite content
	mg/serving	*mg/serving*
DASH food pattern with high-nitrate or high-nitrite food choices (4–5 servings each of vegetables and fruit)		
1 cup raw spinach	926	0.027
1/2 cup cooked collard greens	198	0.06
1/2 cup vegetable juice	42.5	0.02
1 medium banana	6.75	0.014
1/4 cup raisins	1	—
1 medium orange	1	0.02
1/2 cup pomegranate juice	47	0.21
Total	1222	0.351
DASH food pattern with low-nitrate or low-nitrite food choices (4–5 servings each of vegetables and fruit)		
1 cup raw leaf lettuce	103	0.17
1/2 cup broccoli	25	0.09
1/2 cup vegetable juice	42.5	0.02
1 medium apple	0.40	0.01
1/4 cup raisins	1	—
1/2 cup canned fruit cocktail	1	0.1
1/2 cup orange juice	1	0.02
Total	174	0.41

[1]Analysis of foods reflects the mean value from triplicate analyses. Nitrate and nitrite concentrations were quantified by ion chromatography (ENO20 Analyzer; Eicom, Kyoto, Japan).

process of setting DRIs for nutrients considers a broad range of physiologic factors, including nutritional status and potential toxicities. Rational methodologies such as these, including the consideration of normal dietary consumption patterns of nitrate- and nitrite-containing foods, have not been applied in setting exposure limits or in considering the potential health benefits of dietary nitrates and nitrites.

Whereas accidental toxic exposures of nitrates and nitrites have occurred (75), the health risks due to excessive nitrate and nitrite consumption appear only in specific subgroups of the population. The permissible concentration of nitrate in drinking water is 50 mg nitrate/L in the European Union and 44 mg/L in the United States in accordance with World Health Organization recommendations first established in 1970 and reaffirmed in 2004 (76). The US Environmental Protection Agency limits human exposure to inorganic nitrates to >10 mg/L (or 10 ppm nitrate nitrogen) and nitrites to 1 ppm nitrite nitrogen (77). The Joint Food and Agricultural Organization/World Health Organization has set the Acceptable Daily Intake (ADI) for the nitrate ion at 3.7 mg/kg body wt and for the nitrite ion at 0.06 mg/kg body wt (66). Likewise, Environmental Protection Agency has set a Reference Dose for nitrate of 1.6 mg nitrate nitrogen · kg body $wt^{-1} \cdot d^{-1}$ (equivalent to ≈7.0 mg nitrate ion/kg body wt per day).

POTENTIAL CONTEXTS FOR NITRATE- AND NITRITE-ASSOCIATED TOXICITIES

Two types of exposure place susceptible individuals at high risk to the adverse effects of excess nitrite exposure. First, infants younger than 6 mo of age may be exposed to excess nitrates in bacterially contaminated well water, which reduces nitrate to nitrite (78). Infants consuming excess nitrite experience methemoglobinemia or "blue baby syndrome" because of the nitrite-mediated oxidation of ferric (Fe^{2+}) iron in oxyhemoglobin that leads to hypoxia and cyanosis (16, 79). As such, an American Academy of Pediatrics consensus panel concluded that all prenatal and well-infant visits should include questions about the home water supply; if the water source is a private well, the water should be tested for nitrates (80). The panel concluded that infants fed commercially prepared infant foods are generally not at risk of nitrate poisoning, but that home-prepared infant foods from vegetables (eg, spinach, beets, green beans, squash, and carrots) should be avoided until infants are 3 mo of age or older. Breastfed infants are not at risk of excessive nitrate exposure from mothers who ingest water with a high nitrate content (up to 100 ppm nitrate nitrogen) because the nitrate concentration does not increase significantly in breast milk (80).

Note that the few human nitrate and nitrite exposure studies, including children and adults, have not produced methemoglobinemia. Infants exposed to 175–700 mg nitrate/d did not have methemoglobin concentrations >7.5%, which suggests that nitrate alone does not cause methemoglobinemia (81). A more recent randomized 3-way crossover study exposed healthy adults to single doses of sodium nitrite that ranged from 150 to 190 mg per volunteer to 290–380 mg per volunteer (82). The observed methemoglobin concentrations were 12.2% for volunteers receiving the higher dose of nitrite ion and 4.5% for those receiving the lower dose. Recent nitrite infusion studies of up to 110 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 5 min induced methemoglobin concentrations of only 3.2% (45). These data have led scientists to propose alternative explanations for the observed methemoglobinemia in infants, including gastroenteritis and associated iNOS-mediated production of nitric oxide induced by bacteria-contaminated water (83, 84). These studies call into question the mechanistic basis for exposure regulations for nitrate and nitrite. At best, these findings highlight a serious, but context-specific, risk associated with nitrite overexposure in infants.

Experts have questioned the veracity of the evidence supporting the hypothesis that nitrates and nitrites are toxic for healthy adolescent and adult populations (16, 17, 66). It appears that the biologically plausible hypothesis of nitrite toxicity (eg, methemoglobinemia) has essentially transformed a plausible hypothesis into sacrosanct dogma (16), despite the lack of proof (83, 84).

The second context in which nitrate and nitrite exposure has been associated with negative health effects is through the consumption of cured and processed meats (85). Nitrates added to meats serve as antioxidants, develop flavor, and stabilize the red color in meats but must be converted to nitrite to exert these actions. Sodium nitrite is used as a colorant, flavor enhancer, and antimicrobial agent in cured and processed meats. Nitrate and nitrite use in meat products, including bacon, bologna, corned beef, hot dogs, luncheon meats, sausages, and canned and cured meat and hams is subject to limits put forth in Food and Drug Administration (FDA) and US Department of Agriculture (USDA) regulations. These regulations can be found in the Code of Federal Regulations (CFR) (21CFR 170.60, 172.170, and 172.175 for FDA and 9CFR 318.7 for USDA regulations, respectively).

Consumption of red and processed meats is associated with an increased risk of certain types of cancer and chronic obstructive pulmonary disease (85–89). On the basis of the association with cancer risk, the American Institute for Cancer Research's Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective contains the following recommendation "Limit consumption of red meats (such as beef, pork and lamb) and avoid processed meats" (90). A systematic review indicated that up to ≈500 g (≈18 oz) weekly of red meat can be consumed without cancer risk. However, review panelists could not determine a safe consumption level for processed meat; cancer risk was shown to increase with any consumption of processed meats based on a meta-analysis of cohort studies showing an increased risk of colorectal cancer with increased intakes of processed meats (summary estimate of relative risk per 50 g/d: 1.21; 95% CI: 1.04, 1.42) (92). It is worth noting that nitrite or nitrate is not added to fresh meats. A discussion of this association in the context of nitrate and nitrite consumption and gastric physiology is warranted to illuminate the processes relevant to this association.

Direct evidence of the participation of nitrate and nitrite in human carcinogenesis is lacking, despite extensive epidemiologic and animal studies (84). Rodent toxicological studies (91) and human epidemiologic investigations have not shown an unequivocal relation between nitrite exposure and the risk of cancer (71). It is reasonable to conclude that all food sources of nitrate and nitrite are not equal with regard to potential health benefits or risks. The association between nitrite consumption and gastrointestinal cancers was bolstered by findings that ingested nitrites may react with secondary amines or *N*-alkylamides to generate carcinogenic *N*-nitroso compounds (NOCs) (71). Although NOCs have been shown in animal models to be carcinogenic (92), proof in humans has been scant. The *N*-nitrosamides and *N*-nitrosoureas have been shown to be direct mutagens, whereas *N*-nitrosoamines do not act as direct mutagens but generally require activation by microsomal enzymes within the body, perhaps by cytochrome P450 enzymes (93). The use of nitrites in bacon must be accompanied by the use of either sodium erythorbate or sodium ascorbate (vitamin C), antioxidants that inhibit the nitrosation effect of nitrites on secondary amines (94). The use of these antioxidants, along with lower nitrate and nitrite levels in processed meats, has lowered residual nitrite levels in cured meat products in the US by ≈80% since the mid-1970s (95).

A recent study has yielded new insights into the ability of vitamin C to modulate the formation of carcinogenic NOCs under conditions simulating the proximal stomach during the digestion of foods such as processed meats (96). Nitrite in processed meats may be converted to nitrosating species and NOCs by acidification in the presence of thiocyanate at low gastric pH. The formation of NOCs was examined under these conditions in the presence and absence of vitamin C and lipid. In the absence of lipid, vitamin C prevented the formation of *N*-nitrosodiethylamine and *N*-nitrosopiperidine and decreased the formation of *N*-nitrosodimethylamine and *N*-nitrosomorpholine 5-fold and 1000-fold, respectively. In the presence of 10% lipid (a food matrix component for processed meats), the presence of vitamin C increased the formation of nitrosodimethylamine, nitrosodiethylamine, and *N*-nitrosopiperidine 8-, 60-, and 140-fold, respectively. Thus, the presence of lipid converts vitamin C from inhibiting to promoting acid nitrosation. This effect is attributable to the ability of vitamin C to assist in the generation of nitric oxide in the aqueous phase, which enables the regeneration of nitrosating species by reacting with oxygen in the lipid phase (96). Whereas these data require confirmation in animal models and in humans, it provides a biologically plausible mechanism for the observed association between processed meat consumption and gastrointestinal cancer risk. Others have postulated that gastric formation of NOCs may be inhibited by nutrients and other components of vegetables and fruit (97). Clearly, more research is needed to address the potential mechanisms by which certain NOCs are related to cancer risk.

ESTIMATING HUMAN NITRATE AND NITRITE EXPOSURE LEVELS

The recent demonstration of the vasoprotective, blood pressure–lowering, and antiplatelet aggregation properties of nitrite alone, or of nitrite originating from dietary nitrate, suggests that a reexamination of the health effects of dietary sources of nitrate and nitrite would be beneficial (31, 32, 46). An illustrative example of human exposure to nitrate, nitrite, and nitric oxide will serve to support the apparent safety of these exposure levels. Based on an estimated daily intake of 0.77 mg nitrite, nitric oxide production would equate to 11.1 μmol/d, and an intake of 76 mg nitrate would equate to 894 μmol/d or roughly 1 mmol NO_x/d from diet. A 70-kg individual produces 1.68 mmol nitric oxide/d (based on 1 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ nitric oxide production) through the endogenous L-arginine pathway. Notably, the amount of nitrite and nitrate consumed as dietary nitrate and nitrite results in nitric oxide production approximately equal to endogenous sources if, as discussed above, we assume most of the endogenous nitric oxide goes to stepwise oxidation to nitrite and nitrate. Therefore, up to 50% of human steady state concentrations of nitrite and nitrate, which are routinely used as clinical biomarkers of nitric oxide activity, are derived from from dietary sources. Assuming 50 μmol/L nitrite in saliva and a daily production of up to 1.5 L saliva/d, the total nitrite exposure from saliva alone is 75 μmol, or 5.18 mg. The enterosalivary concentration and circulation of nitrate and ultimately nitrite provides an essential pathway for health and host defense (98). If nitrite were, indeed, a carcinogen, we would be advised to avoid swallowing because saliva contains 50–100 μmol/L nitrite, which can increase to near millimolar levels (99) after a nitrate-rich meal. Even more convincing, studies of natives in the high altitude of Tibet have shown that increasing nitrite and nitrate concentrations within the body is a natural physiologic response that is not associated with harmful physiologic effects (100). These data show that normal physiologic exposure levels of nitrite and nitrate greatly exceed concentrations considered to produce health risks. These observations render as questionable the rationale supporting these regulatory limits.

DIETARY CONSUMPTION OF NITRATES AND NITRITES RELATIVE TO WHO ACCEPTABLE DAILY INTAKES

The WHO ADI for nitrate (0–3.7 mg/kg) translates into an equivalent of 222 mg nitrate for a 60-kg adult. Our calculations above indicate that an individual following a DASH dietary pattern with high-nitrate vegetable and fruit choices represented in our convenience sample would exceed this ADI by ≈550%. In

fact, as has been observed previously and confirmed here, a portion of spinach commonly consumed in one serving of salad can exceed the ADI for nitrate (51). The fact that typical consumption patterns of vegetables and fruit exceed regulatory limits for dietary nitrates calls into question the rationale behind current nitrate and nitrite regulations. The physiologic basis for regulating human consumption of plant foods containing nitrates and nitrites should be reevaluated to include potential health benefits.

CONCLUSIONS

The DASH diet forms the basis for public dietary health recommendations in the United States (eg, MyPyramid.gov) and is widely recommended by private health agencies, such as the American Heart Association (101). Taken together, the data considered here support the conclusions of the European Food Safety Authority (66) that benefits of vegetable and fruit consumption outweigh any perceived risk of developing cancer from the consumption of nitrate and nitrite in these foods. Note that the nitrate and nitrite concentrations measured in our convenience sample may differ from samples taken from more disparate geographic locations. We conclude that the data on nitrate and nitrite contents of vegetables and fruit bolster the strength of existing evidence to recommend their consumption for health benefits.

Despite the demonstration of physiologic roles for nitrate and nitrite in vascular and immune function, food sources of nitrates and nitrites as healthful dietary components have received little attention (18). The questionable practice of causal inference with regard to the etiologic roles of dietary nitrates and nitrites in methemoglobinemia and cancer has exerted a detrimental effect on research supporting the health benefits of nitrate- and nitrite-containing foods. This has occurred despite the observed benefits of nitrate and nitrite in medical therapeutics (102). Indeed, data from observational epidemiologic and human clinical studies support the hypothesis that nitrates and nitrites of plant origin play essential physiologic roles in supporting cardiovascular health and gastrointestinal immune function. We support the recent call for a multidisciplinary and systematic review of the biological consequences of dietary nitrate and nitrite consumption (84). The strength of the evidence linking the consumption of nitrate- and nitrite-containing plant foods to beneficial health effects supports the consideration of these compounds as nutrients.

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Effects of Chronic Dietary Creatine Feeding on Cardiac Energy Metabolism and on Creatine Content in Heart, Skeletal Muscle, Brain, Liver and Kidney

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M. Horn, S. Frantz, H. Remkes, A. Laser, B. Urban, A Mettenleiter, K. Schnackerz, S. Neubauer. Effects of Chronic Dietary Creatine Feeding on Cardiac Energy Metabolism and on Creatine Content in Heart, Skeletal Muscle, Brain, Liver and Kidney. *Journal of Molecular and Cellular Cardiology* (1998) 30, 277–284. Little is known about the regulation of total creatine concentration in heart, skeletal muscle, brain, liver and kidney in response to increased dietary creatine intake. The phosphorylated fraction of intracellular creatine (phosphocreatine) remain relatively constant, and therefore, higher intracellular creatine levels may increase the energy reserve of the heart [phosphocreatine and phosphoryl transfer via creatine kinase (CK)] and of other organs. To test the effect of supplying exogenous creatine on the myocardial energy reserve and on creatine content of various organs, rats were given chow containing 0 (Untreated), 1, 3, 5, or 7% (of diet weight) creatine for ~40 days. Thereafter, hearts were perfused and left ventricular developed pressure and heart rate were recorded. High-energy phosphate concentrations were determined with ^{31}P-NMR spectroscopy, CK reaction velocity by ^{31}P-magnetization transfer. Total creatine was determined in heart, skeletal muscle, brain, liver, kidney and serum by high-performance liquid chromatography (HPLC). Creatine feeding increased serum creatine by 73% (1% creatine), 142% (3%), 166% (5%) and 202% (7%). In the heart, increased serum creatine levels did not affect mechanical function; ATP, phosphocreatine, inorganic phosphate, CK reaction velocity and total creatine were all unchanged. Total creatine also remained constant in brain and skeletal muscle, while creatine content increased 4.6-fold in the liver and 1.9-fold in the kidney. We conclude that myocardial energy reserve via CK cannot be increased by exogenous creatine treatment.

Key Words: Creatine metabolism; Cardiac energy reserve; ^{31}P-NMR spectroscopy; Isolated rat heart; ATP; Phosphocreatine.

Introduction

The energy reserve of the heart is determined by myocardial ATP and phosphocreatine concentrations as well as by the rate and extent of ATP transfer via the creatine kinase reaction (see Ingwall, 1993, for a review). The myocardial ATP concentration is one of the most tightly regulated biochemical entities. To date, all interventions have failed to achieve an increase of ATP concentrations above normal levels, presumably since mitochondrial ATP production is subject to close feedback inhibition (Lehninger, 1982). On the other hand, it has not been systematically tested whether interventions exist that can increase myocardial phosphocreatine stores as well as energy reserve

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via creatine kinase to supernormal values. By increasing the myocardial energy reserve, such interventions may have beneficial effects and could thus be of potential clinical interest.

Relatively little is known about the regulation of myocardial phosphocreatine and total creatine stores; creatine kinase kinetics (Neubauer *et al.*, 1988, 1995) and creatine transporter function (Guimbal and Kilimann, 1993; Loike *et al.*, 1988; Odoom *et al.*, 1996) seem to be the major determinants of myocardial creatine and phosphocreatine concentrations [see Wallimann *et al.*, 1992; Wyss and Wallimann, 1994, for a review]. The most obvious intervention that might increase myocardial energy reserve is supply of the heart with increased extracellular creatine concentrations. Preliminary work on skeletal muscle energy metabolism suggests that total creatine content of human skeletal muscle can be increased by 17% with dietary creatine supplementation (Harris *et al.*, 1992). We decided to systematically study this question for the heart, and, in addition, for skeletal muscle, brain, liver and kidney, by chronically providing rats with large amounts of dietary creatine over a wide dosage range, leading to a three-fold increase of blood creatine levels. We report that myocardial phosphocreatine and creatine stores as well as energy reserve via creatine kinase are all unaffected in the presence of increased extracellular creatine concentrations.

Materials and Methods

Animals and isolated heart preparation

All experiments were performed according to the guidelines of the American Physiological Society. Wistar rats weighing ca 250 g were used (Charles River, Sulzfeld, Germany) kept in a 12-h light–dark cycle. Rats were anesthetized by injecting 50 mg pentobarbital sodium intraperitoneally. After thoracotomy, the heart was rapidly excised and immersed in ice-cold buffer. The aorta was dissected free, and mounted onto a cannula attached to a perfusion apparatus, as previously described (Neubauer *et al.*, 1990). Retrograde perfusion of the heart was started in the Langendorff mode at a constant temperature of 37°C and a constant coronary perfusion pressure of 100 mmHg. A small vent made out of polyethylene tubing was pierced through the apex of the left ventricle for drainage of flow from Thebesian veins. For perfusion, phosphate-free Krebs–Henseleit buffer was used containing (mM): NaCl (118), KCl (4.7), $CaCl_2$ (1.75), $MgSO_4$ (1.2), ethylenediaminetetra-acetate tetrasodium (0.5), $NaHCO_3$ (25.0) and glucose (11.0). Equilibrating the buffer with 95% O_2–5% CO_2 yielded a pH of 7.4. Coronary flow was measured by an ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY, USA) built into the perfusate inflow tubing. As previously shown, the perfusion system allowed maintenance of hearts in a steady state for at least 90 min with change of less than 5% for all mechanical and metabolic parameters (Neubauer *et al.*, 1990). For measurement of cardiac performance, a water-filled latex balloon was inserted into the left ventricle through an incision in the left atrial appendage, via the mitral valve, and secured by a ligature. The balloon was connected to a Statham P23Db pressure transducer (Gould Instruments, Glen Burnie, MD, USA) with a small-bore polyethylene tubing for continuous measurement of left ventricular pressure and heart rate on a 4-channel recorder (Graphtec Corp., Tokyo, Japan). Performance was estimated as heart rate (min^{-1}) and left ventricular developed pressure (mmHg).

^{31}P-NMR spectroscopy

The perfused hearts were placed into a 20 mm NMR sample tube and inserted into a probe seated in the bore of a superconducting super-wide-bore (150 mm) 7.05 Tesla magnet (Bruker, Rheinstetten, Germany) as previously described (Horn *et al.*, 1996; Illing *et al.*, 1996; Neubauer *et al.*, 1995). Hearts were bathed in their own perfusate which was pumped from the NMR tube at a level immediately above the heart. An Aspect 3000 computer (Bruker) was used in the pulsed Fourier transform mode to generate ^{31}P-NMR spectra at 121.50 MHz. A 14-channel Shim Unit served to homogenize the magnetic field. Single ("one pulse") spectra were accumulated over 5-min periods, averaging data from 152 free induction decays obtained using a pulse time of 37.6 μs, a pulse angle of 45° and an interpulse delay of 1.93 s. The resonance areas corresponding to ATP, phosphocreatine, inorganic phosphate, monophosphate esters and NAD, which are proportional to the number of phosphorus atoms of the respective compound were measured using the Aspect Integration Program. Relative saturation factors for each resonance were determined by comparing spectra to fully relaxed spectra obtained using a pulse angle of 45° and an interpulse delay of 15 s; correction factors were 1.12 ± 0.03 for phosphocreatine and 1.08 ± 0.05

for inorganic phosphate ($n=15$); spectra were corrected accordingly. In each heart, the area of the [γ-P]-ATP resonance of the first spectrum obtained under control conditions was arbitrarily set to 100% and used as the reference value for all resonances in the sequence of ^{31}P-NMR spectra obtained for the protocol. Absolute ATP concentrations were determined by comparison of peak areas with an external ATP standard. Intracellular pH (pH_i) was measured by comparing the chemical shift between inorganic phosphate and phosphocreatine with values obtained from a standard curve (Moon and Richards, 1973).

^{31}P-NMR magnetization transfer measurements of creatine kinase kinetics

For magnetization transfer experiments each broadband pulse was preceded by a low-power, narrowband pulse at the resonance frequency of [γ-P] ATP for 0 or 3.6 s as previously described (Neubauer *et al.*, 1995). Recycle times for each scan were kept constant at 5.0 s. Separate studies (Neubauer *et al.*, 1995) showed that the narrowband pulse directly attenuated the phosphocreatine magnetization by less than 5% when the carrier frequency was placed 2.5 ppm downfield from the resonance of phosphocreatine. For each pair of saturation transfer spectra, 64 scans were accumulated by repetitively cycling through the two different times of presaturation. Thus, any metabolic deterioration occurring during the saturation transfer measurement was equally distributed among the spectra. A saturation transfer experiment was acquired in 12 min. Stability of the preparation was assessed by comparing one-pulse spectra obtained before and after each magnetization transfer experiment. Magnetization transfer measurements of the forward CK reaction, phosphocreatine $\rightarrow$ [γ-P] ATP, were analyzed according to the two-site chemical exchange model of Forsen and Hoffman (1963), providing estimates of the pseudo first-order rate constant (k_{for}). This assumes that phosphocreatine T_1 values were as previously determined [3.45 ± 0.14 s (Neubauer *et al.*, 1995)] and were unchanged, which is the case after ischemia and reperfusion (Neubauer *et al.*, 1988) and which should also hold true for the conditions of our experiments where cardiac function and high- and low-energy phosphate metabolite concentrations were unaltered after creatine feeding. In addition, in preliminary experiments, direct phosphocreatine T_1 measurements were performed in rats after feeding 3% creatine for $\sim$40 days using saturation transfer as previously described (Neubauer *et al.*, 1995) indicating unchanged values (3.56 ± 0.17 s; $n=4$). Briefly, as the time of saturation at [γ-P] ATP, t, is either 0 or 3.6 s, the integrated signal intensity of the phosphocreatine resonance peak, M_t, corresponds to M_0 and M_∞ (defined as magnetization at zero and infinite saturation times, respectively). k_{for} was then calculated by solving the equation: $k_{for}=M_0 / (M_\infty - T_1) - 1/T_1$. Multiplying the rate constant by substrate concentration yielded reaction velocity.

Measurement of total creatine

At the end of the experiment, hearts were rapidly freeze-clamped using Wollenberger tongues (for this reason, heart weight could not be recorded) as previously described (Neubauer *et al.*, 1995; Neubauer and Ingwall, 1989). Skeletal muscle, brain, liver and kidney were excised, rinsed and also rapidly freeze-clamped. Whole organ homogenates were analyzed for total creatine content. Frozen tissue was powdered in a stainless steel percussion mortar cooled in liquid nitrogen. The powder was homogenized in 0.4 N perchloric acid at 0°C and aliquots of the homogenate were removed for protein determination. The homogenate was neutralized and centrifuged for 5 min. The supernatant was used for measuring total creatine by high pressure liquid chromatography (HPLC) as previously described (Neubauer *et al.*, 1995; Neubauer and Ingwall, 1989). Non-collagen protein was measured by the method of Lowry *et al.* (1951). Tissue creatine concentrations were expressed as nmol/mg protein, i.e. were related to non-collagen ("Lowry") protein content. Blood was centrifuged, and serum creatine concentrations were expressed in mmol/l.

Experimental groups and protocols

Five groups of rats were studied: rats were fed creatine-free chow (fish protein replaced by soya bean; Altromin, Lage, Germany) to which 0% (control; $n=15$), 1% ($n=12$), 3% ($n=18$), 5% ($n=15$) and 7% ($n=17$) creatine was added (% refers to the total weight of chow) for $\sim$40 (39 ± 1) days. After isolation, hearts were given a 15 min stabilization period, during which end-diastolic pressure was set to 10 mmHg by adjusting balloon volume. One 5 min "one-pulse" ^{31}P-NMR spectrum

Table 1 Body weights, coronary flow and cardiac function after 40 days of creatine feeding

Dietary creatine (%)	0	1	3	5	7
n	15	12	18	15	17
Body weight (g)	456±10	433±7	454±10	410±9*	397±7*
LVDP (mmHg)	80±6	77±5	90±4	85±4	89±6
Heart rate (1/min)	321±4	298±9	280±8	301±4	303±7
Coronary flow (ml/min)	23±1	21±4	21±1	23±1	22±1

$P<0.05$, 7% *v* 0% (ANOVA).
LVDP = left ventricular developed pressure.

was then recorded. A saturation transfer measurement of CK reaction velocity followed. Finally, another 5 min "one-pulse" ^{31}P-NMR spectrum was recorded to test for metabolic stability during the saturation transfer measurement. At the completion of the protocol, hearts were freeze-clamped with Wollenberger tongues for determination of total creatine content. In addition, at the time of sacrifice, blood, brain, liver and kidney were collected for determination of creatine content. Skeletal muscle was not saved in the original series of experiments. Therefore, additional experiments were performed: seven rats were fed 0% and eight rats 3% creatine-containing diet for 40 days. Thereafer, animals were sacrificed and skeletal muscle tissue was excised, rinsed and rapidly freeze-clamped with liquid nitrogen.

Statistical analysis

Results from the five groups of rats were compared using a factorial ANOVA (Zar, 1974) with significant differences between individual groups detected by Scheffe's *F*-test (Zar, 1974).

Results

Body weight and cardiac performance

Data given in Table 1 demonstrate that body weights were unchanged after ~40 days of feeding with 1 and 3% dietary creatine. However, 5 and 7% creatine in the diet led to a small reduction of body weights by 10 and 13% after the creatine feeding period. Thus, overall growth and feeding behavior was unaffected by a diet with up to 3% dietary creatine content. Data from Table 1 also demonstrate that chronic creatine feeding did not affect cardiac performance and coronary flow. Left ventricular developed pressure, heart rate and coronary flow were all similar for untreated and the various treated groups; no significant differences were detected for any variable. In addition, hearts from all groups were hemodynamically stable with changes of less than 5% for any functional parameter over the entire length of the protocol (~30 min; data not shown).

Cardiac energy metabolism

Figure 1 shows typical ^{31}P-NMR spectra of an untreated heart and of a heart from the group fed 7% dietary creatine. Clearly, the spectra do not exhibit major differences. Table 2 gives the results of NMR measurements of myocardial ATP levels, phosphocreatine/ATP-ratios, inorganic phosphate/ATP-ratios, intracellular pH, rate constant of the forward creatine kinase reaction and creatine kinase reaction velocity (flux) for the various groups of hearts. The data demonstrate that chronic creatine feeding did not affect myocardial ATP, phosphocreatine or inorganic phosphate levels. Likewise, intracellular pH did not change significantly. At the same time, creatine kinase kinetics remained unchanged, with both the rate constant and creatine kinase reaction velocity being similar for all groups of hearts.

Total creatine content in serum, heart, brain, skeletal muscle, liver and kidney

By increasing dietary creatine content from 0 to 1, 3, 5 and 7% of the diet weight, blood creatine concentrations increased from 0.62±0.14 to 1.07±0.12*, 1.49±0.30*, 1.64±0.10* and 1.87±0.16* mM, respectively (*Scheffe's *F*-test *v* 0%). Thus, creatine supplemented with the diet was taken up by the intestine leading to an up to

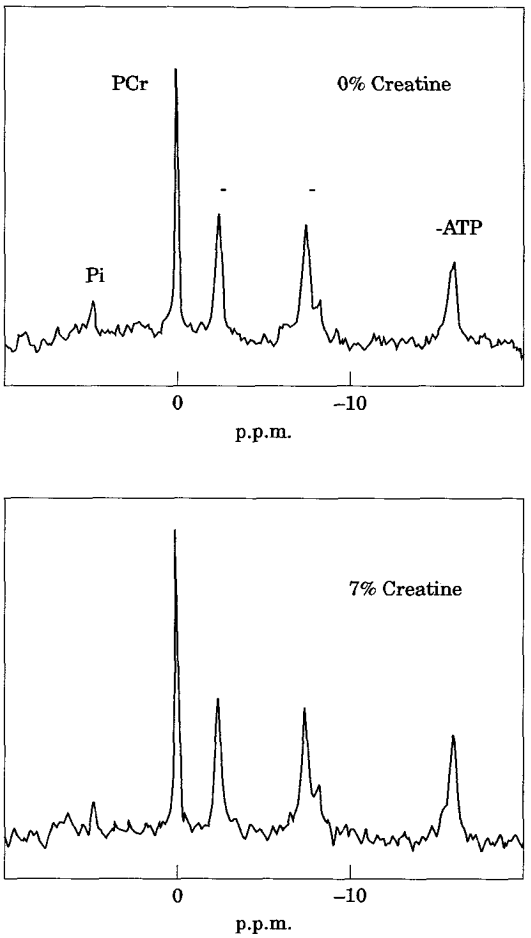

Figure 1 Typical ^{31}P-NMR spectra of perfused hearts isolated from an untreated (0% creatine) and a creatine-treated (7% creatine) rat. The spectra demonstrate that no major alterations of high-energy phosphate content occur with creatine feeding. For experimental details see methods. P_i = inorganic phosphate; PCr = phosphocreatine; γ-, α- and β-[P]-atom of ATP.

~three-fold increase of blood creatine concentrations. Figure 2 gives the results of biochemical analysis of total creatine content in heart, brain, liver and kidney. In spite of substantially increased blood creatine concentrations, total creatine content in the myocardium remained unchanged [Fig. 2(a)]. Similarly, total creatine did not increase in brain [Fig. 2(b)], and was also unchanged in skeletal muscle of rats fed 3% creatine for ~40 days (127 ± 8 *v* 119 ± 14 nmol/mg protein in rats fed 0% creatine). In contrast, total creatine increased up to ~five-fold in liver [Fig. 2(c)] and up to ~two-fold in kidney [Fig. 2(d)].

Discussion

Definition of the model

In the present work, we studied the effects of chronically providing large amounts of dietary creatine over a period of ~40 days on creatine content of various organs and on myocardial energy reserve. By feeding 1, 3, 5 and 7% creatine, we achieved up to ~three-fold increases of blood creatine content. Blood creatine increased in a monotonic manner with increasing dietary creatine. At the same time, body weight remained normal with up to 3% creatine feeding, while with 5 and 7%, body weight was progressively reduced by up to 13%. Reduced body weights in the 5 and 7% groups were not due to reduced food intake, since the amount of chow consumed by each group (food was weighed twice every week) was similar (33.6 ± 0.9, 33.9 ± 0.8, 34.0 ± 0.8, 32.3 ± 2.3 and 34.1 ± 1.2 g/day per rat for 0, 1, 3, 5 and 7% creatine groups; no significant differences by ANOVA). This suggests that large amounts of creatine have more fundamental effects on trophic and/or resorptive processes in the mammalian organism. To our knowledge, the effects of such large amounts of dietary and blood creatine levels have not been studied before, and these points certainly warrant further investigation. In any case, the present model allows the generation of blood creatine levels three-fold higher than normal, and defines the dose-dependence of dietary *v* blood creatine levels.

Table 2 Cardiac energy metabolism after 40 days of creatine feeding

Dietary creatine (%)	0	1	3	5	7
ATP (mM)	10.8 ± 0.4	10.1 ± 0.3	11.6 ± 0.5	10.2 ± 0.5	10.6 ± 0.6
PCr/ATP	1.30 ± 0.06	1.36 ± 0.09	1.28 ± 0.05	1.42 ± 0.05	1.33 ± 0.08
Pi/ATP	0.33 ± 0.04	0.42 ± 0.08	0.40 ± 0.03	0.43 ± 0.09	0.38 ± 0.03
pH_i	7.15 ± 0.01	7.15 ± 0.01	7.14 ± 0.00	7.15 ± 0.00	7.13 ± 0.01
k_{for} (1/s)	0.89 ± 0.04	0.81 ± 0.02	0.94 ± 0.04	0.80 ± 0.03	0.84 ± 0.04
CK flux (mM/s)	11.6 ± 0.5	11.0 ± 0.3	11.6 ± 0.4	11.3 ± 0.5	11.2 ± 0.5

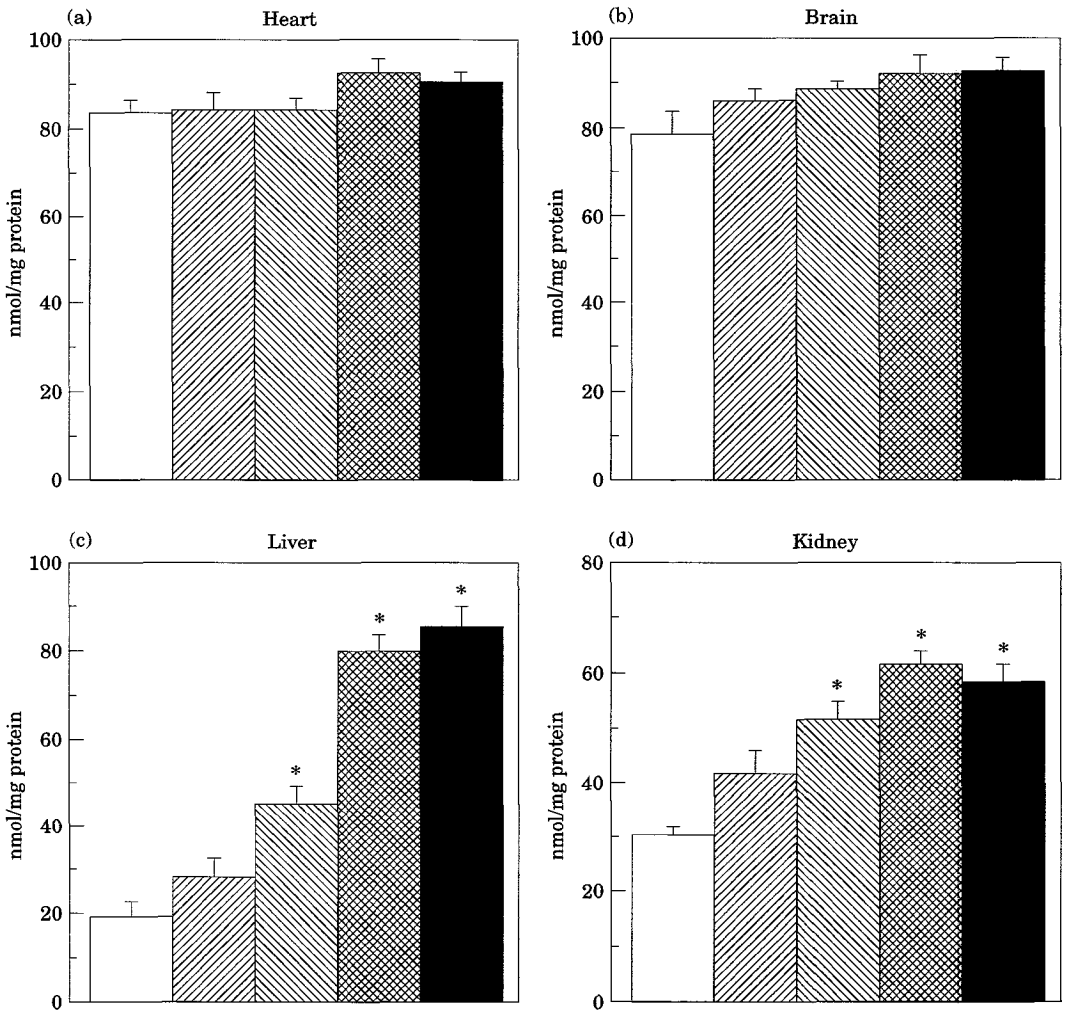

Figure 2 Total creatine content in (a) heart, (b) brain, (c) liver and (d) kidney (nmol/mg protein) determined —40 days after feeding diet with varying amounts of creatine (0, 1, 3, 5 or 7%). Significant differences (1, 3, 5 or 7% *v* 0% Scheffe's *F*-test) are indicated (*). □, 10% creatine; ▨, 1% creatine; ▧, 3% creatine; ▩, 5% creatine; ■, 7% creatine.

Effects of oral creatine supplementation on mechanical function and energy metabolism of the heart

Our data demonstrate that increasing dietary creatine and, thus, extracellular creatine concentrations does not affect cardiac performance or coronary flow at low and even at high diet creatine levels that reduce body weight. Furthermore, our results clearly show that, as the extracellular creatine concentration is varied from normal to up to ~three-fold supernormal values, myocardial creatine, phosphocreatine, inorganic phosphate, intracellular pH and creatine rate constant and reaction velocity all remained unaffected. This also shows that free ADP levels (Lawson and Veech, 1979), calculated from the creatine kinase equilibrium assumption, and the free energy change of ATP hydrolysis are unchanged under these conditions. Taken together, these findings demonstrate that, under normal circumstances, cardiac high-energy phosphate metabolism and energy reserve are unaffected in the presence of three-fold varying extracellular creatine concentrations, showing that in normal heart, energy reserve via CK is not regulated by extracellular creatine concentrations.

Total creatine in serum, heart, brain, skeletal muscle, liver and kidney

Creatine, which is synthesized by the liver, pancreas and kidney, is released into the bloodstream by these

organs [see, Wyss and Wallimann, 1994, for a review] and is taken up by the myocardium, skeletal muscle and brain via a specific creatine transporter protein with a K_m in the range of 20–60 μM (Guimbal and Kilimann, 1993). In cultured human muscle cells it was demonstrated that this creatine transporter undergoes significant up- and down-regulation in the presence of varying extracellular creatine concentrations. Muscle cells maintained in medium containing 1 mM creatine exhibited one-third of the creatine transport activity of cells maintained in creatine-free medium (Loike *et al.*, 1988). In cultured G8 myoblasts, creatine transport was shown to be dependent on Na^+-K^+-ATPase activity and β_2-receptor stimulation, and net cell creatine content was largely independent of extracellular creatine content (Odoom *et al.*, 1995). Although we did not directly determine creatine transporter kinetics in the whole-heart model, our findings of unchanged myocardial, skeletal muscle and brain creatine concentrations in the presence of several-fold increased blood creatine levels strongly suggest that under the *in vivo* conditions of our model, the creatine transporter system of these organs downregulates substantially, thereby preventing an increase of creatine levels that would otherwise occur with increased extracellular creatine concentrations. Our findings of unchanged creatine levels in skeletal muscle stand in contrast with previous work by Harris *et al.* (1992) who showed that total creatine content of human skeletal muscle could be increased by 17% with dietary creatine supplementation. It is unclear at present whether this discrepancy is due to species differences or has methodological reasons; our data, however, do not support the notion that creatine control of normal skeletal muscle can be increased by providing creatine with the diet. As an extension of this work, it would be interesting to study the effects of chronic β_2-receptor stimulation on creatine levels of the various organs in our model; from the work of Odoom *et al.* (1996) it is conceivable that creatine levels might increase under such conditions.

On the other hand, creatine levels did increase significantly in liver and kidney. The mechanisms responsible for such organ-specific differences remain to be determined, but may be related to the absolute creatine levels these tissues show under physiological conditions. Heart, skeletal muscle and brain show high total creatine levels of ~90 nmol/mg protein (heart, brain) and more (skeletal muscle) in the presence of physiological blood creatine concentrations, but do not increase creatine levels further with increased extracellular supply, while liver (18 nmol/mg protein) and kidney (30 nmol/mg protein) both have substantially lower physiological creatine contents, but, with increased serum creatine concentrations, are able to accumulate creatine concentrations that are close to concentrations reached in heart and brain (~90 nmol/mg protein) with normal serum creatine concentrations (85 nmol/mg protein in liver, 58 nmol/mg protein in kidney) (Fig. 2). In follow-up studies it will be interesting to determine creatine transporter activities in all of these organs with and without creatine feeding.

A question our study does not address is whether chronic dietary creatine supplementation can increase creatine levels and energy reserve under conditions when both are substantially reduced, as it is described for animal models of chronic heart failure (Markiewicz *et al.*, 1986; Nascimben *et al.*, 1995; Neubauer *et al.*, 1995; Zhang *et al.*, 1996) and also for the failing human myocardium (Nascimben *et al.*, 1996). Under these conditions, creatine transporter kinetics may show completely different characteristics (Seppet *et al.*, 19855), and the effects of increased blood creatine levels cannot be predicted for this situation. In this context, the work of Zweier *et al.* (1991) is of interest, where perfusion with buffer containing 50 mM creatine had no effect on normal hearts, but acutely increased myocardial phosphocreatine stores by 52% and left ventricular developed pressure by 28% in hearts depleted of creatine by the creatine analogue beta-guanidinobutyric acid. Thus, the effects of creatine treatment under conditions of creatine depletion, such as in heart failure, should be examined in future experimental and clinical studies.

In summary, by chronically providing rats with large amounts of creatine in the diet, serum creatine levels can be increased ~three-fold. This results in an accumulation of creatine in liver and kidney, but not in heart, brain and skeletal muscle. In rat heart, mechanical function and energy reserve via CK are unaffected by increasing serum creatine concentrations. The effects of exogenous creatine in heart failure remain to be studied, however.

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Chronic high-dose creatine feeding does not attenuate left ventricular remodeling in rat hearts post-myocardial infarction

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Abstract

Objective: In heart failure, cardiac energy metabolism is compromised. The failing myocardium is characterized by reduced contents of both phosphorylated (phosphocreatine) and non-phosphorylated (free) creatine content as well as decreased energy reserve via creatine kinase (creatine kinase reaction velocity). These changes may contribute to cardiac dysfunction. The purpose of the present study was to determine whether chronic feeding with high-dose dietary creatine prevents the derangement of energy metabolism and the development of left ventricular remodeling in a rat model of heart failure, i.e. post-myocardial infarction (MI). **Methods and results:** Rats were subjected to sham operation or left coronary artery ligation. Surviving rats were fed with 0% (untreated) or 3% creatine (related to weight of diet) for 8 weeks. Creatine feeding increased serum creatine levels significantly ~2-fold. Thereafter, hearts were isolated, perfused and left ventricular pressure–volume curves obtained. Steady state and dynamic (CK reaction velocity) high-energy phosphate metabolism was determined with ^{31}P NMR spectroscopy. In both MI groups (treated n=8, untreated n=7), pressure–volume curves were shifted right- and downward compared to both sham groups (treated n=5, untreated n=7), i.e. creatine had no effect on left ventricular remodeling. Likewise, similar reductions of phosphocreatine, free creatine and creatine kinase reaction velocity (untreated sham 12.0±0.7 mmol/l×s; untreated MI 7.8±0.7*; treated sham 13.6±1.0; treated MI 7.2±1.1*; * p<0.025 sham vs. MI) were found in both MI groups. **Conclusions:** Chronic creatine feeding of post-MI rats is ineffective in preventing the functional and energetic derangements occurring post-MI. Inspite of increased serum creatine levels, neither the normal nor the failing heart accumulates additional creatine.

Keywords: Creatine; ^{31}P NMR spectroscopy; Heart failure; ATP; Phosphocreatine; Isolated rat heart

1. Introduction

Both experimental [1–3] and clinical studies [4–7] have shown that the hypertrophied and failing myocardium is characterized by a depletion of creatine compounds, i.e. both phosphorylated (phosphocreatine) and unphosphorylated ('free') creatine are decreased. The reduction of phosphocreatine is largely responsible for the substantial decrease of energy reserve via creatine kinase (creatine kinase reaction velocity, or 'flux') in the failing heart [1,2,8]. Although direct proof has been difficult, it is well conceivable that changes in creatine metabolism and creatine kinase (CK) kinetics contribute to contractile dysfunction (see Ref. [9] for a review). Compounds of proven therapeutic benefit in heart failure such as beta-receptor-blockers [10] or angiotensin-converting enzyme inhibitors [2,11] were shown to increase (phospho-) creatine content and CK reaction velocity in experimental heart failure models in parallel with their beneficial functional effects, but these compounds do not primarily act on energy metabolism. Thus, it would be interesting to study interventions that primarily and specifically increase creatine content and, therefore, improve CK kinetics in heart failure, and to test for their possible beneficial

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functional effects. Cardiomyocytes do not synthesize creatine but accumulate the compound against a large concentration gradient through the action of the specific Na^+-creatine cotransporter protein [12]. Studies in athletes have shown that creatine content of skeletal muscle can be increased 15–20% by chronically supplying high dosages of creatine with the diet, and that skeletal muscle work output increases in parallel [13,14], although in rat, this has not been observed [15].

Based on these observations, purpose of the present work was to study the effects of chronically supplying high dietary dosages of creatine on myocardial creatine and phosphocreatine content and on cardiac structure and function in a clinically highly relevant model of heart failure, i.e. the rat heart post-myocardial infarction (MI). If successful, oral creatine might have been a novel approach to the treatment of chronic heart failure. However, our results indicate that, inspite of substantially increased extracellular creatine concentrations, neither the normal nor the failing heart increases its creatine content.

2. Methods

2.1. Animals and experimental myocardial infarction

Infarcts or sham operations were carried out in 12-weeks old Wistar rats as previously described [1,16]. The left coronary artery was ligated after left thoracotomy under ether anesthesia. Mortality rate of infarcted rats for the first 24 h after the operation was 40–50%. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1985).

2.2. Isolated heart preparation

Rats were re-anesthetized by injecting 50 mg pentobarbital sodium intraperitoneally. After thoracotomy, the heart was rapidly excised and immersed in ice-cold buffer. The aorta was dissected free, and mounted onto a cannula attached to a perfusion apparatus, as previously described [17]. Retrograde perfusion of the heart was started in the Langendorff mode at a constant temperature of 37°C and a constant coronary perfusion pressure of 100 mmHg. For perfusion, phosphate-free Krebs–Henseleit buffer was used as described [17]. Coronary flow was measured by an ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY). As previously shown, the perfusion system allowed maintenance of hearts in a steady state for at least 90 min with changes of less than 5% for all mechanical and metabolic parameters [17]. For measurement of cardiac performance, a water-filled latex balloon was inserted into the left ventricle. The balloon was connected to a Statham P23Db pressure transducer (Gould Instruments, Glen Burnie, MD) with a small-bore polyethylene tubing for continuous measurement of left ventricular pressure and heart rate on a MacLab system (version 3.5, AD instruments, Castle Hill, Australia). Left ventricular pressure–volume curves were obtained by increasing balloon volume in a stepwise manner (delta 0.05 ml) until maximum LVDP was reached or until LVEDP exceeded 50 mmHg. Thereafter, LVEDP was set to 10 mmHg for the duration of the NMR measurements. At the end of the protocol, hearts were taken off the cannula, and the right ventricle was cut off and rapidly frozen. The entire left ventricle was fixed in formalin as previously described for histologic determination of infarct size.

2.3. Determination of infarct size

Infarct size was determined by a previously described technique [1]. The left ventricle was embedded in paraffin, and 20-μm sections were cut serially from apex to base of the heart. Sections were stained with Picrosirius Red and were mounted on glass plates, scanned (Nikon EF scanner, Nikon, Japan) and planimetry was performed with the NIH Image software (version 1.59, National Institutes of Health). Lengths of scar and non-infarcted muscle for both endocardial and epicardial surfaces were determined for each section. The ratio of the lengths of scar and surface circumferences defined the infarct size for endo- and epicardial surfaces, respectively. Final infarct size was determined as the average of endo- and epicardial surfaces and is given in percent. All hearts with an infarct size of less than 25% were excluded from the analysis.

2.4. ^{31}P NMR spectroscopy

The perfused hearts were placed into a 20-mm NMR sample tube and inserted into a probe seated in the bore of a superconducting super-wide-bore (150 mm) 7.05 Tesla magnet (Bruker, Rheinstetten, FRG) as previously described [1,11,18]. Hearts were bathed in their own perfusate. An Aspect 3000 computer (Bruker, Rheinstetten, FRG) was used in the pulsed Fourier transform mode to generate ^{31}P NMR spectra at 121.50 MHz. A 14-channel Shim Unit served to homogenize the magnetic field. Single ('one pulse') spectra were accumulated over 5-min periods, averaging data from 152 free induction decays obtained using a pulse time of 37.6 ms, a pulse angle of 45° and an interpulse delay of 1.93 s. The resonance areas corresponding to ATP, phosphocreatine, inorganic phosphate, monophosphate esters and NAD, which are proportional to the number of phosphorus atoms of the respective compound, were measured using the NMR1 software package (Tripos, St. Louis, MO) and were corrected for partial saturation. In each heart, the area of the [γ-P]ATP resonance of the first spectrum obtained under control conditions was arbitrarily set to 100% and used as the reference value for all resonances in the sequence of ^{31}P

NMR spectra obtained for the protocol. Absolute ATP concentrations were previously determined for sham hearts as 10.8±0.8 mmol/l, for residual intact left ventricular tissue of MI hearts as 10.6±0.8 mmol/l [1]. Since the protocol (histologic determination of left ventricular infarct size, cutoff of right ventricle for creatine analysis within ~20 s) did not allow absolute ATP quantification by HPLC, ATP concentrations for sham and MI hearts were assumed to be the same as for the previous study [1]. In addition, it was assumed that chronic creatine feeding does not increase ATP above normal values and does not decrease ATP in sham or MI hearts, both assumptions very likely to be correct: ATP cannot increase above normal levels, since mitochondrial ATP production is subject to close feedback inhibition by ATP; chronic creatine feeding does not decrease myocardial ATP content, as was previously shown [15]. Intracellular pH (pH_i) was measured by comparing the chemical shift between inorganic phosphate and phosphocreatine with values obtained from a standard curve [19].

2.5. ^{31}P NMR magnetization transfer measurements of creatine kinase kinetics

For magnetization transfer experiments each broadband pulse was preceded by a low-power, narrowband pulse at the resonance frequency of [γ-P]ATP for 0 or 3.6 s as previously described [15]. Recycle times for each scan were kept constant at 5.0 s. A saturation transfer experiment was acquired in 12 min. Stability of the preparation was assessed by comparing one-pulse spectra obtained before and after each magnetization transfer experiment. Magnetization transfer measurements of the forward CK reaction, phosphocreatine→[γ-P]ATP, were analyzed according to the two-site chemical exchange model of Forsen and Hoffman [20], providing estimates of the pseudo first-order rate constant (K_{for}). Multiplying the rate constant by substrate concentration yielded reaction velocity [19].

2.6. Measurement of creatine content

At the end of the experiment, the right ventricular free wall was cut off and was rapidly frozen in liquid nitrogen for determination of total creatine content by high pressure liquid chromatography (HPLC) as previously described [15,21]. Free creatine was then calculated for each heart as total creatine minus phosphocreatine. Non-collagen protein was measured by the method of Lowry et al. [22]. In analogy to high-energy phosphate concentrations, free creatine concentrations were expressed in mmol/l, assuming that 50% of wet weight represents intracellular H_2O [23]. Blood was centrifuged, and serum creatine concentrations were measured by HPLC as previously described [15], and were expressed in mmol/l.

2.7. Cytosolic phosphorylation potential

The cytosolic phosphorylation potential (PP; M^{-1}) was calculated as described [24] as:

$$PP = ATP: (ADP \times \text{inorganic phosphate})$$

where ADP (μmol/l) is

$$(ATP \times \text{creatine}): (\text{phosphocreatine} \times H^+ \times K_{eq}),$$

H^+ is the intracellular hydrogen ion concentration, and K_{eq} is the equilibrium constant of the creatine kinase reaction (1.66×10^{-9} M^{-1}) [1].

2.8. Experimental groups and protocols

Four groups of rats were studied: Sham operated untreated (Sh; $n=7$), infarcted untreated (MI; $n=7$), sham operated treated (Sh+Cr; $n=5$) and infarcted treated (MI+Cr; $n=8$). After sham operation or MI, rats were randomized to receive creatine-free chow (fish protein replaced by soy bean; Altromin, Lage, FRG), to which 0% (untreated) or 3% (creatine treated) creatine (Sigma, Deisenhofen, FRG) was added (% refers to the total weight of chow) for a period of 8 weeks. We had previously shown that this dose of creatine achieves a maximum increase in serum creatine levels without reducing body weight [15]. Food intake was similar (32.4±0.5 g/day) for all four groups. After 8 weeks, hearts were isolated and perfused. Isolated hearts were given a 15-min stabilization period, during which end-diastolic pressure was set to 10 mmHg by adjusting balloon volume. Thereafter, pressure–volume curves were obtained as described above. Hearts were allowed to restabilize at EDP=10 mmHg. One 5-min 'one-pulse' ^{31}P NMR spectrum was then recorded. A saturation transfer measurement of CK reaction velocity followed. Finally, another 5-min 'one-pulse' ^{31}P NMR spectrum was recorded to test for metabolic stability during the saturation transfer measurement. At the completion of the protocol, hearts were saved as described for determination of infarct size and measurement of creatine content. In addition, at the time of sacrifice, blood was collected and centrifuged for determination of serum creatine levels.

2.9. Statistical analysis

Results were compared using an unpaired, Bonferroni-corrected *t*-test [25]. The following comparisons between groups were made: Sh vs. Sh+Cr, Sh vs. MI, MI vs. MI+Cr and Sh+Cr vs. MI+Cr. With a maximum of two comparisons per group (e.g. Sham vs. MI, Sham vs. Sham+Cr), p values <0.025 were considered to indicate statistical significance.

Table 1
Characteristics of treated and untreated, sham and MI hearts[a]

	Sh	MI	Sh+Cr	MI+Cr
n	7	7	5	8
MI size (%)	0	36±1	0	37±2
Heart weight (g)	1.84±0.06	2.14±0.10	1.98±0.07	2.25±0.20
Body weight (g)	529±31	555±17	589±11	559±17
HW/BW (‰)	3.47±0.26	3.85±0.13	3.38±0.17	3.99±0.36
HR (1/min)	256±8	231±17	253±17	254±16
CF (ml/min)	30.3±1.4	23.3±2.1[b]	29.0±1.0	28.9±1.2
CF/HW (ml/min/g)	17.2±1.4	12.6±1.1	14.8±1.1	15.6±2.0

[a] Infarct size (MI size), heart weight/body weight ratio (HW/BW), heart rate (HR), coronary flow (CF); values for coronary flow/heart weight (CF/HW) are normalized for mass of perfused tissue.
[b] $p<0.025$ Sham vs. MI.

3. Results

3.1. Infarct size, heart and body weights

Table 1 shows infarct sizes, heart weights, body weights and their ratios for the four experimental groups. Infarct size was similar for creatine-treated and untreated infarcted animals (36 vs. 37%). Inspite of the loss of almost 40% of left ventricular tissue due to infarction, marginally increased heart weights and heart weight/body weight ratios in both MI groups attest to hypertrophy of residual intact myocardial tissue due to remodeling. Creatine feeding of MI hearts did not change heart weights or heart weight/body weight ratios.

3.2. Mechanical performance and coronary flow

Fig. 1 shows pressure–volume curves for left ventricular volume and developed pressure from all experimental groups. As it is typical for this model, infarcted groups showed a displacement of the pressure–volume curve towards higher volumes (dilatation) and lower developed pressures (dysfunction). During chronic creatine feeding, pressure–volume curves did not change significantly for both sham and MI hearts. In addition, Table 1 shows that heart rate (recorded at EDP = 10 mmHg) was similar for all experimental groups. Therefore, creatine feeding did not affect cardiac function or structure in normal or in chronically failing heart. Table 1 also shows changes in coronary flow. Both in absolute ($p<0.025$) and relative ($p<0.05$) terms, coronary flow was significantly reduced in the untreated MI group. Interestingly, however, this decrease of coronary flow was prevented by chronic creatine feeding of infarcted hearts.

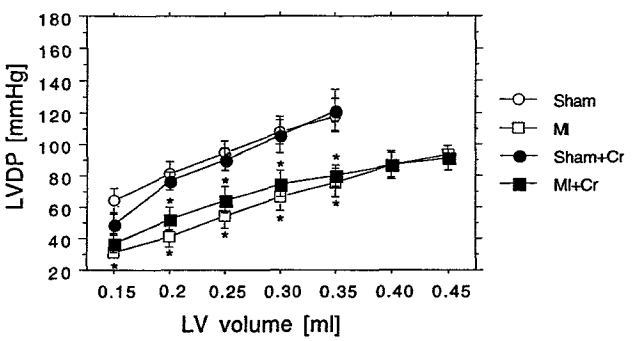

Fig. 1. Pressure–volume curves for left ventricular developed pressure. The curves for both infarcted groups show a right- and downward displacement, attesting to left ventricular dilatation and dysfunction. Creatine feeding was without effect in both sham and MI hearts.

3.3. Energy metabolism in sham and infarcted, treated and untreated hearts

Fig. 2 shows typical ^{31}P NMR spectra from the four experimental groups. The spectra demonstrate the reduction of the phosphocreatine resonance area that occurs post-MI. This was observed for both untreated and creatine-treated animals. Table 2 shows mean metabolite concentrations for the experimental groups. ATP concentrations were assumed to be as previously described (see Section 2); ATP levels remain constant in this heart failure model. Creatine feeding did not alter phosphocreatine content in sham hearts, and phosphocreatine levels were reduced to 81% in untreated MI and to 83% in treated MI groups; similarly, free creatine levels were reduced to 65 and 74%, respectively. Thus, chronic creatine feeding did not significantly alter the contents of phosphorylated and non-phosphorylated creatine in sham and in MI hearts. Also, cytosolic phosphorylation potentials were not significantly different among groups. Inorganic phosphate (P_i) was low in all experimental groups, the statistically significant increase of P_i in creatine-fed MI hearts was small in absolute terms (+1.4 mmol/l). Intracellular pH was similar for all groups.

Table 2 also shows results of saturation transfer measurements of CK kinetics. Compared to sham hearts, where creatine feeding had no effect, CK reaction velocity ('energy reserve via CK') decreased by 35% in untreated and by 53% in treated MI groups. Thus, creatine feeding did not increase CK reaction velocity in either sham and MI hearts. Therefore, our data demonstrate that chronic creatine feeding had no effect on cardiac function and steady state as well as dynamic high-energy phosphate

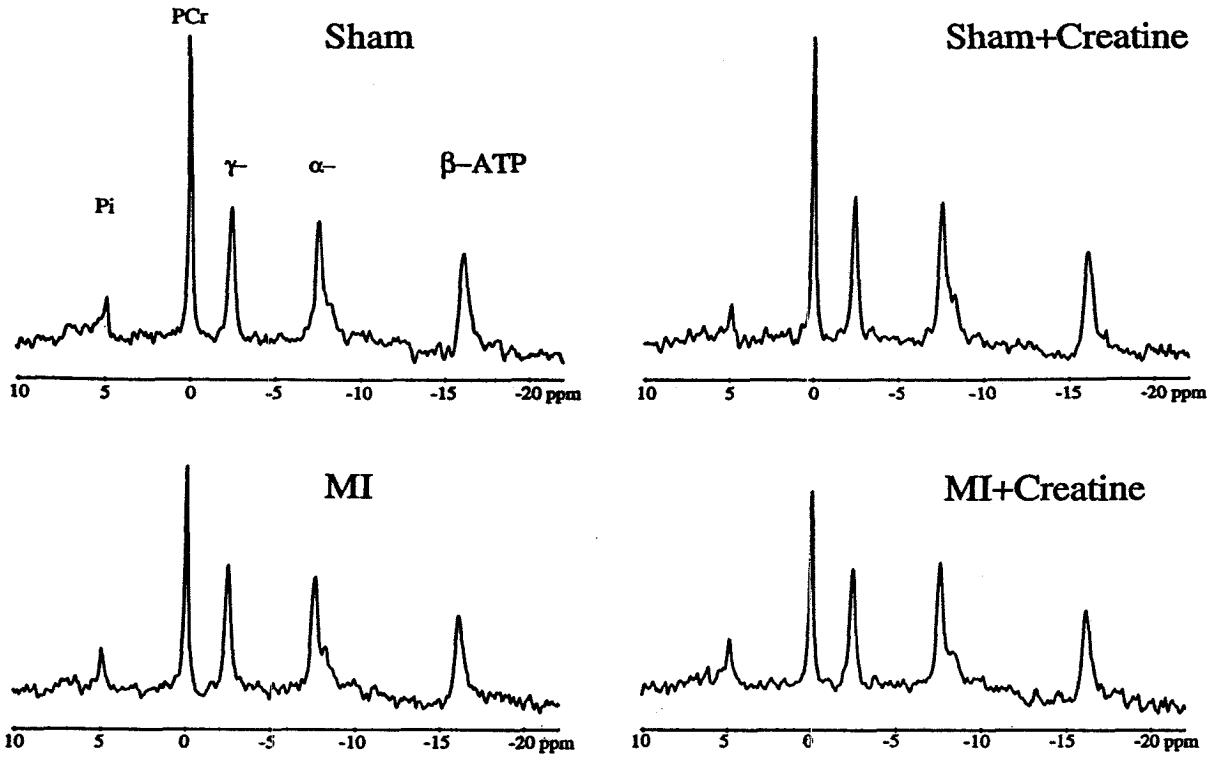

Fig. 2. Typical ^{31}P NMR spectra for sham operated (upper left), infarcted (lower left), creatine-treated sham-operated (upper right) and creatine-treated infarcted (lower right) hearts. The figure shows the reduction of the phosphocreatine resonance area in both infarcted groups (bottom row). Creatine feeding did not increase phosphocreatine content in sham or infarcted hearts.

metabolism in both normal and failing hearts. Serum creatine levels were not significantly different between sham and MI hearts and were increased ~2-fold in both creatine-treated groups.

4. Discussion

4.1. Definition of the model

In the present study, we examine possible beneficial effects of chronic creatine feeding on cardiac function and energy metabolism in a well-established model of heart failure, i.e. in the rat heart post-myocardial infarction. Using this model, we have previously defined left ventricular dilatation, systolic and diastolic dysfunction, as they occur 2 months after coronary artery ligation in the isolated heart model [1,10,26], as well as in vivo [27]. In residual intact myocardium, ATP levels remain unchanged [1], phosphocreatine and free creatine levels decrease by up to 40% [1,16] and the creatine kinase reaction velocity is reduced by ~50% [1]. All these changes could be

Table 2
Steady-state concentrations of high- and low-energy phosphates, cytosolic phosphorylation potential, intracellular pH, creatine kinase reaction velocity and serum creatine content[a]

	Sh	MI	Sh+Cr	MI+Cr
ATP (mmol/l)	Set to 10.8	Set to 10.6	Set to 10.8	Set to 10.6
PCr (mmol/l)	14.2±1.7	11.5±0.8[b]	15.5±1.2	12.9±0.5[b]
Free Cr (mmol/l)	15.5±0.7	10.0±0.8[b]	15.1±0.8	11.1±1.0[b]
P_i (mmol/l)	2.5±0.4	2.7±0.2	2.8±0.4	4.2±0.3[b,c]
pH_i	7.15±0.01	7.17±0.01	7.16±0.00	7.14±0.02
CK flux (mmol/l×s)	12.0±0.7	7.8±0.7[b]	13.6±1.0	7.2±1.1[b]
PP (10^3 M^{-1})	37.5±5.3	39.4±1.2	36.6±4.0	31.5±2.2
Cr_{serum} (mmol/l)	0.54±0.10	0.43±0.08	1.00±0.11[c]	0.88±0.10[c]

[a] Phosphocreatine (PCr), creatine (Cr), inorganic phosphate (P_i), creatine kinase reaction velocity (CK flux), cytosolic phosphorylation potential (PP). ATP concentrations were assumed to be as previously determined by HPLC (10.8 mmol/l in sham and 10.6 mmol/l in MI; see Section 2).
[b] $p<0.025$ Sham vs. MI.
[c] $p<0.025$ treated vs. untreated.

reproduced for the MI group in the present study. We also previously showed that pharmacologic interventions such as angiotensin-converting-enzyme inhibitors [28] or beta-receptor blockers [10] were able to fully or partially reverse the functional and energetic deterioration occurring after MI. Thus, we studied a heart failure model where functional and energetic derangements are well defined and where beneficial pharmacologic interventions can be demonstrated to afford protection.

Our approach to increase myocardial creatine and, thus, as it was hoped, phosphocreatine levels, was to chronically increase the extracellular creatine supply to the heart by feeding high dosages of creatine with the diet for 2 months. In the present work, 3% creatine feeding increased serum creatine levels significantly ~2-fold in both sham and MI groups. At the same time, body weights were unaltered by creatine feeding. Thus, we tested the effect of chronically doubling the extracellular creatine supply to the heart on the development of mechanical and energetic derangement following coronary ligation.

4.2. Effects of oral creatine supplementation on mechanical function and energy metabolism in heart failure

Whether changes in high-energy phosphate metabolism contribute to contractile dysfunction in heart failure is an old and still unresolved question [9]. Although direct proof is missing, it is conceivable that reduction of energy reserve via creatine kinase and failure to maintain a high cytosolic phosphorylation potential and free energy change of ATP hydrolysis (ΔG) [24] are mechanisms contributing to cardiac failure. In the present work, we attempted to prevent the decrease of phosphocreatine and free creatine in heart failure by increasing creatine supply to the heart. In case this intervention were successful, we could test whether maintenance of high (phospho-) creatine content has beneficial functional effects.

In the mammalian organism, creatine is synthesized by liver and kidney and, in addition, is supplied with a non-vegetarian diet (see Ref. [29] for a review). Cardiomyocytes lack the enzymes required for creatine synthesis and, instead, accumulate creatine via a specific membrane protein, the Na^+-creatine cotransporter, which has recently been cloned [12], against a large concentration gradient [12,29]. Intracellularly, creatine is phosphorylated to phosphocreatine via creatine kinase. The degradation of phosphocreatine and creatine occurs non-enzymatically via spontaneous decarboxylation and passive transmembrane diffusion [12], processes unlikely to be regulated. Therefore, the obvious approach to attempt to increase intracellular creatine content is to increase the substrate supply to the creatine transporter by raising extracellular creatine levels. Previous work has shown that in human skeletal muscle, this approach is successful: Here, total creatine content can be increased by 17% with dietary creatine supplementation [13]. Species differences may exist, however, since in rat, skeletal muscle creatine content could not be increased by creatine feeding [15]. In skeletal muscle, a 17% increase of creatine levels has functional relevance, and maximum work output increases significantly (see Ref. [14] for a review). Based on these observations, it was conceivable that the same approach would also be effective for cardiac muscle. Although it was shown before that normal cardiac muscle does not increase its creatine content with increased serum creatine levels [15], it was possible that this intervention is effective for conditions where creatine is chronically depleted. Furthermore, the regulation of myocardial creatine uptake and degradation in heart failure is still completely unknown. We know of no published clinical study of the effects of creatine treatment in heart failure patients, but there are some initial studies related to beneficial effects of phosphocreatine treatment (e.g. [30,31]), where acute and subacute improvement of cardiac function was observed. However, the creatine transporter does not transport phosphocreatine, and no other mechanism for direct uptake of the polar compound phosphocreatine by cardiomyocytes has been demonstrated. Thus, if phosphocreatine does have a beneficial effect in heart failure, it is not related to improvement of cardiac energetics but, likely, rather due to some indirect (vascular, electrophysiologic or other) effect.

The results presented here unequivocally demonstrate that the strategy to chronically provide high dosages of creatine to the failing heart is ineffective in preventing the decrease of creatine content and of energy reserve via creatine kinase. Inspite of chronically doubling extracellular creatine concentrations, myocardial phosphorylated and non-phosphorylated creatine levels as well as creatine kinase reaction velocity all remained unaffected. In light of these findings, it was not surprising that creatine feeding had no effect on functional consequences of left ventricular remodeling post-MI with the exception of maintenance of coronary flow. Since heart creatine levels could not be increased with higher serum creatine levels, we could not test the hypothesis that maintaining high cardiac creatine levels during the development of heart failure has beneficial functional effects. Therefore, inspite of a lack of effect of creatine feeding, our results do not argue against a causal role of energy metabolism in heart failure, but also do not provide new evidence in support of this concept.

We can only speculate on the reasons why higher extracellular creatine levels are ineffective in increasing creatine levels and mechanical work in the normal or failing heart. There are currently no data available on the regulation of creatine transport under in vivo conditions, and speculations have to rely on results obtained from cell culture models, none of which have used cardiomyocytes from failing hearts. Creatine transporter kinetics were shown to be dependent on Na/K-ATPase activity, $beta_2$-receptor stimulation and thyroid hormone status [32], but

none of these mechanisms is likely to bear on our studies. One possible explanation, however, is that both in human muscle cells as well as in G8 myoblasts, creatine transporter activity was inversely related to extracellular creatine supply, i.e. was downregulated by high and upregulated by low extracellular creatine concentrations [33]. Thus, the most likely explanation for the failure of dietary creatine to increase heart creatine stores is that the creatine transporter activity is downregulated under these circumstances, thereby preventing an increase of intracellular creatine concentrations. In our study, creatine transporter levels were not directly determined, and it will be important to do this in future studies in order to understand the regulation of this transporter in normal and diseased heart. A second explanation for the lack of effect of creatine feeding could be that in cell culture, creatine uptake is saturated at ~500 μM and can, thus, be increased no further by increasing extracellular creatine concentrations from ~500 to ~1000 μM, corresponding to serum levels of untreated and treated groups in our study. It is unknown, however, whether saturating concentrations are different for normal and failing heart. Whatever the reasons for a lack of effect, our study demonstrates that providing high extracellular creatine concentrations is not an effective treatment approach in heart failure in terms of improving myocardial function. Thus, other strategies to increase creatine content in heart failure will have to be developed. Specific stimulators of creatine transport are currently unknown. One approach that may be effective is to increase creatine transporter protein content, either via gene transfer or transgenic overexpression models. These studies remain to be done.

An unexpected finding was that creatine feeding prevented the small but significant decrease of coronary flow occurring post-MI, while coronary flow in sham hearts was unaffected by creatine. It is unlikely that this can be explained by an acute vasodilator effect of creatine, since hearts were perfused under creatine-free conditions, and since in sham operated hearts, creatine did not alter coronary flow. In addition, perfusion of isolated rat hearts with 1 mmol/l creatine did not acutely affect coronary flow ($n=3$, data not shown). It is, thus, conceivable that chronic creatine feeding has effects on the coronary microcirculation. Data to this point are currently lacking, and this observation may warrant further study. Whatever the reasons, however, maintenance of higher global coronary flow levels by creatine feeding in MI hearts did not have any beneficial functional or energetic consequences. Furthermore, inorganic phosphate levels were slightly, yet significantly, higher in creatine-treated MI hearts. The reason for this is unclear, but the small absolute magnitude of the change (+1.4 mmol/l) and the fact that the amount of total NMR observable phosphates was the same for all groups (data not shown) suggest that this finding does not have physiological significance. In addition, cytosolic phosphorylation potential did not change significantly, again arguing against a pathophysiological role for the increase of inorganic phosphate in creatine-treated MI hearts.

In summary, we have shown that chronically providing failing rat hearts with increased extracellular creatine fails to elicit beneficial structural, functional and energetic effects. The additional creatine is not accumulated by the heart. Thus, creatine treatment does not seem to be a promising approach for the treatment of heart failure.

Acknowledgements

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CREATINE
CASRN: 57-00-1

For other data, click on the Table of Contents

Human Health Effects:

Drug Warnings:
Phosphocreatine inhibits enzymes in the glycolitic pathway, including glyceraldehydes-3-phosphate dehydrogenase, phosphofructokinase and pyruvate kinase.
[Medical Economics Co; Physicians Desk Reference for Nutritional Supplements 1st ed p.115 (2001)] **PEER REVIEWED**

Safety data are lacking and are urgently needed, especially for long-term use of **creatine** and for use among the pediatric population (including adolescence) and among those in poor health. There are some reports that long-term use of **creatine** may be nephrotoxic. This needs further investigation before long-term **creatine** supplementation can be recommended under any circumstance.
[Medical Economics Co; Physicians Desk Reference for Nutritional Supplements 1st ed p.116 (2001)] **PEER REVIEWED**

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[Medical Economics Co; Physicians Desk Reference for Nutritional Supplements 1st ed p.115 (2001)] **PEER REVIEWED**

Probable Routes of Human Exposure:
Occupational exposure to **creatine** may occur through dermal contact with this compound at workplaces where **creatine** is produced or used. Increased exposure to **creatine** among the general population may be limited to those administered the drug, a nutritional supplement. Intentional human exposure may have occurred from **creatine** use as a possible performance enhancement drug in athletes(1).
[(1) Donahue JL, Lowenthal DT; Am J Ther 7: 365-73 (2000)] **PEER

REVIEWED** PubMed Abstract

Body Burden:
The mean **creatine** level in the urine of 50 patients with muscular dystrophy was 3.56 mmol/L which is much higher than that of the controls 0.24 mmol/L(1). The mean level of **creatine** in the blood of patients with uremia was 0.1 mmol/L which is over twice that of the controls blood level of 0.04 mmol/L(1).
[(1) Cone MV et al; National Body Burden Database. Chemicals Identified in Human Biological Media. Vol 7, Part 1,2,3. Washington, DC: US EPA, EPA-560-5-84-003 (1986)] **PEER REVIEWED**

Emergency Medical Treatment:

Emergency Medical Treatment:

EMT Copyright Disclaimer:

The following Overview, *** **CREATINE** ***, is relevant for this HSDB record chemical.

Life Support:

- This overview assumes that basic life support measures have been instituted.

Clinical Effects:

0.2.1 SUMMARY OF EXPOSURE
0.2.1.1 ACUTE EXPOSURE
A) At the time of this review, there is no acute overdose data available for **creatine** supplements. This review is based on limited reports of possible toxicity following supplementation with **creatine**.

B) Transient renal dysfunction was reported in one patient with a history of medically managed renal disease.
C) The US FDA has received reports of adverse events (gastrointestinal effects, seizures, behavioral changes, headache, fatigue, and possible anaphylactoid reaction) associated with the use of **creatine** supplements. NOTE: This information is limited to a voluntary reporting system and in many cases the adverse events occurred in patients using more than one nutritional product. Causality is difficult to assess.
D) Anecdotal reports describe muscle cramps, heat intolerance, muscle strain, and gastrointestinal effects (stomach cramps, nausea, vomiting, and diarrhea) associated with **creatine** use.
E) Water retention commonly occurs during initial **creatine** supplementation and may explain the weight gain observed in users.
F) Although **creatine** supplements are popular among athletes, the elderly may also consume **creatine** based on its purported ability to limit muscle atrophy.

0.2.3 VITAL SIGNS

0.2.7 NEUROLOGIC

0.2.7.1 ACUTE EXPOSURE

A) Several anecdotal reports of seizure have occurred. Other CNS effects reported have included: nervousness, anxiety, headache, and fatigue.

0.2.8 GASTROINTESTINAL

0.2.8.1 ACUTE EXPOSURE

A) Reports of abdominal cramps, nausea and vomiting, and diarrhea have occurred.

0.2.10 GENITOURINARY

0.2.10.1 ACUTE EXPOSURE

A) Transient renal dysfunction was reported in one patient following **creatine** supplements. Elevated urinary creatinine levels could be anticipated during **creatine** supplementation. Based on this additional workload to the kidneys, supplementation is not recommended in patients with a history of renal dysfunction.

0.2.12 FLUID-ELECTROLYTE

0.2.12.1 ACUTE EXPOSURE

A) Water retention has been reported during initial **creatine** supplementation.

0.2.15 MUSCULOSKELETAL

0.2.15.1 ACUTE EXPOSURE

A) Muscle cramps have been reported.

0.2.19 IMMUNOLOGIC

0.2.19.1 ACUTE EXPOSURE

A) **Creatine** supplements may be associated with anaphylaxis.

0.2.20 REPRODUCTIVE HAZARDS

A) At the time of this review, no data were available to assess the potential effects of exposure to this agent during pregnancy or lactation.

Laboratory:

A) Monitor renal function following a significant acute exposure or chronic use.

B) Monitor fluid status as indicated in symptomatic patients.
C) Monitor neurological function as indicated in symptomatic patients.

Treatment Overview:

0.4.2 ORAL EXPOSURE
A) At the time of this review, no overdose data is available.
B) Treatment is SYMPTOMATIC and SUPPORTIVE.
C) ACTIVATED CHARCOAL: Administer charcoal as a slurry (240 mL water/30 g charcoal). Usual dose: 25 to 100 g in adults/adolescents, 25 to 50 g in children (1 to 12 years), and 1 g/kg in infants less than 1 year old.
D) SEIZURES: Administer a benzodiazepine IV; DIAZEPAM (ADULT: 5 to 10 mg, repeat every 10 to 15 min as needed. CHILD: 0.2 to 0.5 mg/kg, repeat every 5 min as needed) or LORAZEPAM (ADULT: 2 to 4 mg; CHILD: 0.05 to 0.1 mg/kg).
1) Consider phenobarbital or propofol if seizures recur after diazepam 30 mg (adults) or 10 mg (children > 5 years).
2) Monitor for hypotension, dysrhythmias, respiratory depression, and need for endotracheal intubation. Evaluate for hypoglycemia, electrolyte disturbances, hypoxia.

Range of Toxicity:

A) At the time of this review, a toxic dose for **creatine** has not been established.
B) Although no toxic level has been established, it has been postulated that daily doses of more than 2 grams or 0.03 grams/kilogram might cause renal dysfunction.

[Rumack BH POISINDEX(R) Information System Micromedex, Inc., Englewood, CO, 2010; CCIS Volume 146, edition expires May, 2010. Hall AH & Rumack BH (Eds): TOMES(R) Information System Micromedex, Inc., Englewood, CO, 2010; CCIS Volume 146, edition expires May, 2010.] **PEER REVIEWED**

Animal Toxicity Studies:

Non-Human Toxicity Excerpts:

/LABORATORY ANIMALS: Chronic Exposure or Carcinogenicity/ Chronic **creatine** supplementation in rats down-regulates **creatine** transporter protein expression. ... Leading to lower amounts entering cell at any given time.
[Medical Economics Co; Physicians Desk Reference for Nutritional Supplements 1st ed p.115 (2001)] **PEER REVIEWED**

Metabolism/Pharmacokinetics:

Metabolism/Metabolites:
Within muscle and nerve cells, about 60 to 67% of the **creatine** entering the cells gets converted to phosphocreatine via the enzyme **creatine** kinase. About 2% of **creatine** is converted to creatinine, and both **creatine** and creatinine are excreted by the kidneys.
[Medical Economics Co; Physicians Desk Reference for Nutritional Supplements 1st ed p.115 (2001)] **PEER REVIEWED**

Absorption, Distribution & Excretion:
In muscle and nerve, most of the **creatine** is phosphorylated to phosphocreatine (pCr) in a reaction that is catalyzed by the enezyme **creatine** kinase (CK), there are three isoforms of (isoenzymes) of CK. CK-MM is the skeletal muscle isoform; CK-BB, the brain isoform, and CK-MB, the isoform found in cardiac muscle. Most of the PCr in the body is in skeletal muscle.
[Medical Economics Co; Physicians Desk Reference for Nutritional Supplements 1st ed p.115 (2001)] **PEER REVIEWED**

Creatine is absorbed from the small intestines and enters the portal circulation and is transported to the liver. The ingested **creatine**, and the **creatine** made in the liver, is then transported into the systemic circulation and distributed into various tissues of the body, including muscle and nerves, by crossing the cell membrane via a specific **creatine**-transported system against a 200:1 gradient.
[Medical Economics Co; Physicians Desk Reference for Nutritional Supplements 1st ed p.115 (2001)] **PEER REVIEWED**

Mechanism of Action:
Supplemental **creatine** may have an energy-generating action during anaerobic exercise and may also have neuroprotective and cardioprotective actions.
[Medical Economics Co; Physicians Desk Reference for Nutritional Supplements 1st ed p.115 (2001)] **PEER REVIEWED**

Creatine, **creatine** kinase and phosphocreatine make up an intricate cellular enegy buffering and transport system connecting sites of energy production in the mitochondria with sites of energy consumption. CK is a key enzyme in involved in cellular energy homeostasis. It reversibly catalyzes the transfer of the high-energy phosphate bond in PCr to adenosine diphosphate (ADP) to form adenosine triphosphate (ATP), and it catalyzes the transfer of the high-energy phosphate bond in ATP to **creatine** to form PCr. During periods of intense exercise and skeletal muscle contraction, bioenergetic metabolism switches from one in which oxidative phosphorylation is the major pathway of ATP production to one in which so-called anaerobic glycolysis becomes dominant.
[Medical Economics Co; Physicians Desk Reference for Nutritional Supplements 1st ed p.115 (2001)] **PEER REVIEWED**

Pharmacology:

Therapeutic Uses:
Creatine, as well as a **creatine** analogue called cyclocreatine, inhibit growth of a broad range of solid tumors in rat models of cancer; these tumors express high levels of CK. Although the mechanism for tumor inhibition is unknown...
[Medical Economics Co; Physicians Desk Reference for Nutritional Supplements 1st ed p.115 (2001)] **PEER REVIEWED**

Drug Warnings:
Phosphocreatine inhibits enzymes in the glycolitic pathway, including glyceraldehydes-3-phosphate dehydrogenase, phosphofructokinase and pyruvate kinase.
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[Medical Economics Co; Physicians Desk Reference for Nutritional Supplements 1st ed p.115 (2001)] **PEER REVIEWED**

Bionecessity:
Creatine is a non-protein amino acid found in animals, in much lesser amounts, plants. **Creatine** is synthesized in the kidney, liver and pancreas from the amino acids L-arginine, glycine and L methionine. Following its biosynthesis, **creatine** is transported to the skeletal muscle, heart, brain and other tissues. Most of the **creatine** in these tissues is metabolized to phosphocreatine (**creatine** phosphate). Phosphocreatine is a major energy storage form in the body.

[Medical Economics Co; Physicians Desk Reference for Nutritional Supplements 1st ed p.114 (2001)] **PEER REVIEWED**

Environmental Fate & Exposure:

Environmental Fate/Exposure Summary:

Creatine's production and use as diagnostic in myocardial disfunctions and as a performance enhancing supplement may result in its release to the environment through various waste streams. **Creatine** is produced by the liver, pancreas and kidney for the use of transferring phosphate from adenosine triphosphate (ATP). **Creatine** is predominantly found in skeletal and cardiac muscle. If released to air, an estimated vapor pressure of 7.9X10-4 mm Hg at 25 deg C indicates **creatine** will exist solely in the vapor phase in the atmosphere. Vapor-phase **creatine** will be degraded in the atmosphere by reaction with photochemically-produced hydroxyl radicals; the half-life for this reaction in air is estimated to be 4 hours. **Creatine** does not absorb light at wavelengths greater than 290 nm and therefore should not be susceptible to direct photolysis. If released to soil, **creatine** is expected to have very high mobility based upon an estimated Koc of 24. The pKa values for the amino and carboxylic acid moieties of **creatine** are estimated to be 12.7 and 3.8, respectively, indicating that this compound will primarily exist as a zwitter ion and zwitter ions do not volatilize from water or moist soil surfaces. **Creatine** is not expected to volatilize from dry soil surfaces based upon its vapor pressure. If released into water, **creatine** is not expected to adsorb to suspended solids and sediment based upon the estimated Koc. **Creatine** has been shown to be degraded by activated sludge. An estimated BCF of 3 suggests the potential for bioconcentration in aquatic organisms is low. Hydrolysis is not expected to be an important environmental fate process since this compound lacks functional groups that hydrolyze under environmental conditions. Occupational exposure to **creatine** may occur through dermal contact with this compound at workplaces where **creatine** is produced or used. Increased exposure to **creatine** among the general population may be limited to those administered the drug, a nutritional supplement. Intentional human exposure may have occurred from **creatine** use as a possible performance enhancement drug in athletes. (SRC)
PEER REVIEWED

Probable Routes of Human Exposure:

Occupational exposure to **creatine** may occur through dermal contact with this compound at workplaces where **creatine** is produced or used. Increased exposure to **creatine** among the general population may be limited to those administered the drug, a nutritional supplement. Intentional human exposure may have occurred from **creatine** use as a possible performance enhancement drug in athletes(1).
[(1) Donahue JL, Lowenthal DT; Am J Ther 7: 365-73 (2000)] **PEER REVIEWED** PubMed Abstract

Body Burden:

The mean **creatine** level in the urine of 50 patients with muscular dystrophy was 3.56 mmol/L which is much higher than that of the controls 0.24 mmol/L(1). The mean level of **creatine** in the blood of patients with uremia was 0.1 mmol/L which is over twice that of the controls blood level of 0.04 mmol/L(1).
[(1) Cone MV et al; National Body Burden Database. Chemicals Identified in Human Biological Media. Vol 7, Part 1,2,3. Washington, DC: US EPA, EPA-560-5-84-003 (1986)] **PEER REVIEWED**

Natural Pollution Sources:
Creatine is produced by the liver, pancreas, and kidney for the use of transferring phosphate from adenosine triphosphate (ATP). **Creatine** is predominantly found in skeletal and cardiac muscle(1).
[(1) O'Neil MJ, ed; The Merck Index. 13th ed. Whitehouse Station, NJ: Merck and Co., Inc. p. 449 (2001)] **PEER REVIEWED**

Artificial Pollution Sources:
Creatine's production and use as a diagnostic for myocardial disfunction(1) and as a performance enhancing supplement(2) may result in its release to the environment through various waste streams(SRC).
[(1) O'Neil MJ, ed; The Merck Index. 13th ed. Whitehouse Station, NJ: Merck and Co., Inc. p. 449 (2001) (2) Donahue JL, Lowenthal DT; Am J Ther 7: 365-73 (2000)] **PEER REVIEWED**

Environmental Fate:
TERRESTRIAL FATE: Based on a classification scheme(1), an estimated Koc value of 24(SRC), determined from a water solubility of 1.33X10+4 mg/L(2) and a regression-derived equation(3), indicates that **creatine** is expected to have very high mobility in soil(SRC). The pKa values for the amino and carboxylic acid moieties of **creatine** are estimated to be 12.7 and 3.8(4), respectively, indicating that this compound will primarily exist as a zwitter ion and zwitter ions do not volatilize from moist soil surfaces. **Creatine** is not expected to volatilize from dry soil surfaces(SRC) based upon an estimated vapor pressure of 7.9X10-4 mm Hg(SRC), determined from a fragment constant method(5). **Creatine** has been reported to be degraded by activated sludge(6).
[(1) Swann RL et al; Res Rev 85: 17-28 (1983) (2) Yalkowsky SH, He Y, eds; Handbook of aqueous solubility data. Boca Raton, FL: CRC Press p. 124 (2003) (3) Lyman WJ et al; Handbook of Chemical Property Estimation Methods. Washington, DC: Amer Chem Soc pp. 4-5 (1990) (4) Hilal SH et al; pp. 291-353 in Quantitative Treatments of Solute/Solvent Interactions: Theoretical and Computational Chemistry Vol. 1 NY, NY: Elsevier (1994). SPARC pKa/property server available at http://ibmlc2.chem.uga.edu/sparc/ as of Jun 20, 2005. (5) Lyman WJ; p. 31 in Environmental Exposure From Chemicals Vol I, Neely WB, Blau GE, eds, Boca Raton, FL: CRC Press (1985) (6) Ebisuno T, Takimoto M; Eisei Kagaku 27: 156-62 (1981)] **PEER REVIEWED**

AQUATIC FATE: Based on a classification scheme(1), an estimated Koc value of 24(SRC), determined from a water solubility of 1.33X10+4 mg/L(2) and a regression-

derived equation(3), indicates that **creatine** is not expected to adsorb to suspended solids and sediment(SRC). The pKa values for the amino and carboxylic acid moieties of **creatine** are estimated to be 12.7 and 3.8, respectively(4), indicating that this compound will primarily exist as a zwitter ion and zwitter ions do not volatilize from water surfaces. According to a classification scheme(5), an estimated BCF of 3(SRC), determined from its water solubility(2) and a regression-derived equation(3), suggests the potential for bioconcentration in aquatic organisms is low(SRC). **Creatine** has been shown to be degraded by activated sludge(7).
[(1) Swann RL et al; Res Rev 85: 17-28 (1983) (2) Yalkowsky SH, He Y, eds; Handbook of aqueous solubility data. Boca Raton, FL: CRC Press p. 124 (2003) (3) Lyman WJ et al; Handbook of Chemical Property Estimation Methods. Washington, DC: Amer Chem Soc pp. 4-5, 5-5 (1990) (4) Hilal SH et al; pp. 291-353 in Quantitative Treatments of Solute/Solvent Interactions: Theoretical and Computational Chemistry Vol. 1 NY, NY: Elsevier (1994). SPARC pKa/property server available at http://ibmlc2.chem.uga.edu/sparc/ as of Jun 20, 2005. (5) Franke C et al; Chemosphere 29: 1501-14 (1994) (6) Meylan WM, Howard PH; J Pharm Sci 84: 83-92 (1995) (6) Meylan WM et al; Environ Toxicol Chem 18: 664-72 (1999) (7) Ebisuno T, Takimoto M; Eisei Kagaku 27: 156-62 (1981)] **PEER REVIEWED**

ATMOSPHERIC FATE: According to a model of gas/particle partitioning of semivolatile organic compounds in the atmosphere(1), **creatine**, which has an estimated vapor pressure of 7.9X10-4 mm Hg at 25 deg C(SRC), determined from a fragment constant method(2), will exist solely in the vapor phase in the atmosphere. Vapor-phase **creatine** is degraded in the atmosphere by reaction with photochemically-produced hydroxyl radicals(SRC); the half-life for this reaction in air is estimated to be 4 hours(SRC), calculated from its rate constant of 9.5x10-11 cu cm/molecule-sec at 25 deg C(SRC) that was derived using a structure estimation method(3). **Creatine** does not absorb light at wavelengths >290 nm(4) and therefore is not expected to be susceptible to direct photolysis by sunlight(SRC).
[(1) Bidleman TF; Environ Sci Technol 22: 361-367 (1988) (2) Lyman WJ; p. 31 in Environmental Exposure From Chemicals Vol I, Neely WB, Blau GE, eds, Boca Raton, FL: CRC Press (1985) (3) Meylan WM, Howard PH; Chemosphere 26: 2293-99 (1993) (4) Lide DR, Milne GW, eds; Handbook of Data on Organic Compounds. 3rd. Boca Raton, FL: CRC Press 3: 2901 (1994)] **PEER REVIEWED**

Environmental Biodegradation:
AEROBIC: **Creatine** has been reported to be aerobically degraded by activated sludge(1).
[(1) Ebisuno T, Takimoto M; Eisei Kagaku 27: 156-162 (1981)] **PEER REVIEWED**

ANAEROBIC: **Creatine**, at a concn of 500 umol/10 ml, was fermented into methane by methanogenic enrichment cultures from Leine River mud (lake Kolksee, Germany) and black pond mud, containing Methanosarcina barkeri(1). **Creatine** was fermented into methane with an efficiency of 1.59 moles of methane/mole of **creatine**(1). **Creatine** did not support growth of a pure culture of M. barkeri(1).
[(1) Hippe H et al; Proc Natl Acad Sci USA 76: 494-498 (1979)] **PEER

REVIEWED** PubMed Abstract

Environmental Abiotic Degradation:
The rate constant for the vapor-phase reaction of **creatine** with photochemically-produced hydroxyl radicals has been estimated as 9.5X10-11 cu cm/molecule-sec at 25 deg C(SRC) using a structure estimation method(1). This corresponds to an atmospheric half-life of about 4 hours at an atmospheric concentration of 5X10+5 hydroxyl radicals per cu cm(1). **Creatine** is not expected to undergo hydrolysis in the environment due to the lack of hydrolyzable functional groups(2). **Creatine** does not absorb at wavelengths >290 nm(3) and therefore is not expected to be susceptible to direct photolysis by sunlight(SRC).
[(1) Meylan WM, Howard PH; Chemosphere 26: 2293-99 (1993) (2) Lyman WJ et al; Handbook of Chemical Property Estimation Methods. Washington, DC: Amer Chem Soc pp. 7-4, 7-5 (1990) (3) Lide DR, Milne GW, eds; Handbook of Data on Organic Compounds. 3rd. Boca Raton, FL: CRC Press 3: 2901 (1994)] **PEER REVIEWED**

Environmental Bioconcentration:
An estimated BCF of 3 was calculated for **creatine**(SRC), using water solubility of 1.33X10+4 mg/L(1) and a regression-derived equation(2). According to a classification scheme(3), this BCF suggests the potential for bioconcentration in aquatic organisms is low.
[(1) Yalkowsky SH, He Y, eds; Handbook of aqueous solubility data. Boca Raton, FL: CRC Press p. 124 (2003) (2) Lyman WJ et al; Handbook of Chemical Property Estimation Methods. Washington, DC: Amer Chem Soc pp. 5-5 (1990) (3) Franke C et al; Chemosphere 29: 1501-14 (1994)] **PEER REVIEWED**

Soil Adsorption/Mobility:
The Koc of **creatine** is estimated as 24(SRC), using a water solubility of 1.33X10+4 mg/L(1) and a regression-derived equation(2). According to a classification scheme(3), this estimated Koc value suggests that **creatine** is expected to have very high mobility in soil.
[(1) Yalkowsky SH, He Y, eds; Handbook of aqueous solubility data. Boca Raton, FL: CRC Press p. 124 (2003) (2) Lyman WJ et al; Handbook of Chemical Property Estimation Methods. Washington, DC: Amer Chem Soc pp. 4-5 (1990) (3) Swann RL et al; Res Rev 85: 17-28 (1983)] **PEER REVIEWED**

Volatilization from Water/Soil:
The pKa values of the amino and carboxy moieties of **creatine** are 12.7 and 3.8(1), respectively, indicating that this compound will primarily exist as a zwitter ion and ions do not volatilize from water or moist soil surfaces. **Creatine** is not expected to volatilize from dry soil surfaces(SRC) based upon an estimated vapor pressure of 7.9X10-4 mm Hg(SRC), determined from a fragment constant method(2).
[(1) Hilal SH et al; pp. 291-353 in Quantitative Treatments of

Solute/Solvent Interactions: Theoretical and Computational Chemistry Vol. 1 NY, NY: Elsevier (1994). SPARC pKa/property server available at http://ibmlc2.chem.uga.edu/sparc/ as of Jun 20, 2005. (2) Lyman WJ; p. 31 in Environmental Exposure From Chemicals Vol I, Neely WB, Blau GE, eds, Boca Raton, FL: CRC Press (1985)] **PEER REVIEWED**

Environmental Standards & Regulations:

Chemical/Physical Properties:

Molecular Formula:

C4-H9-N3-O2

[O'Neil, M.J. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. 13th Edition, Whitehouse Station, NJ: Merck and Co., Inc., 2001., p. 449] **PEER REVIEWED**

Molecular Weight:

131.13

[Lide, DR (ed.). CRC Handbook of Chemistry and Physics. 81st Edition. CRC Press LLC, Boca Raton: FL 2000, p. 3-173] **PEER REVIEWED**

Melting Point:

Decomposes at 303 deg C

[Lewis, R.J., Sr (Ed.). Hawley's Condensed Chemical Dictionary. 13th ed. New York, NY: John Wiley & Sons, Inc. 1997., p. 309] **PEER REVIEWED**

Density/Specific Gravity:

1.33 at 25 deg C

[Lide, DR (ed.). CRC Handbook of Chemistry and Physics. 81st Edition. CRC Press LLC, Boca Raton: FL 2000, p. 3-173] **PEER REVIEWED**

Dissociation Constants:

pKa1 = 3.8 (carboxylic acid); pKa2 = 12.7 (amine)

[Hilal SH et al; pp. 291-353 in Quantitative Treatments of Solute/Solvent Interactions: Theoretical and Computational Chemistry Vol. 1 NY, NY: Elsevier (1994). SPARC pKa/property server Available from, as of June 20, 2005: http://ibmlc2.chem.uga.edu/sparc/] **PEER REVIEWED**

Octanol/Water Partition Coefficient:

log Kow = -3.72 (est)

[US EPA; Estimation Programs Interface (EPI). ver. 3.11. U.S. EPA

version for Windows. Washington, DC: U.S. EPA (2003). Available from, as of Apr 27, 2005: http://www.epa.gov/opptintr/exposure/docs/episuite.htm] **PEER REVIEWED**

pH:

Neutral reaction to litmus

[O'Neil, M.J. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. 13th Edition, Whitehouse Station, NJ: Merck and Co., Inc., 2001., p. 449] **PEER REVIEWED**

Solubilities:

Slightly soluble in water, insoluble in ether

[Lewis, R.J., Sr (Ed.). Hawley's Condensed Chemical Dictionary. 13th ed. New York, NY: John Wiley & Sons, Inc. 1997., p. 309] **PEER REVIEWED**

Slightly soluble in ether and ethanol

[Lide, DR (ed.). CRC Handbook of Chemistry and Physics. 81st Edition. CRC Press LLC, Boca Raton: FL 2000, p. 3-173] **PEER REVIEWED**

In water, 1.33X10+4 mg/L at 18 deg C

[Yalkowsky, S.H., He, Yan., Handbook of Aqueous Solubility Data: An Extensive Compilation of Aqueous Solubility Data for Organic Compounds Extracted from the AQUASOL dATAbASE. CRC Press LLC, Boca Raton, FL. 2003., p. 124] **PEER REVIEWED**

Spectral Properties:

MASS: 76453 (NIST/EPA/MSDC Mass Spectral Data Base, 1990 Version)

[Lide, D.R., G.W.A. Milne (eds.). Handbook of Data on Organic Compounds. Volume I. 3rd ed. CRC Press, Inc. Boca Raton ,FL. 1994., p. v3: 2901] **PEER REVIEWED**

IR: 21934 (Sadtler Research Laboratories IR prism collection)

[Lide, D.R., G.W.A. Milne (eds.). Handbook of Data on Organic Compounds. Volume I. 3rd ed. CRC Press, Inc. Boca Raton ,FL. 1994., p. V3: 2901] **PEER REVIEWED**

UV max = 215 nm

[Lide, D.R., G.W.A. Milne (eds.). Handbook of Data on Organic Compounds. Volume I. 3rd ed. CRC Press, Inc. Boca Raton ,FL. 1994., p. V3: 2901] **PEER REVIEWED**

Vapor Pressure:

7.9X10-4 mm Hg at 25 deg C (est)

[US EPA; Estimation Programs Interface (EPI). ver. 3.11. U.S. EPA version for Windows. Washington, DC: U.S. EPA (2003). Available from, as of Apr 27, 2005:

http://www.epa.gov/opptintr/exposure/docs/episuite.htm] **PEER REVIEWED**

Other Chemical/Physical Properties:

Crystals heat of fusion = -537.2, Molar heat capacity = 140.9 J/mole-K
[Lide, DR (ed.). CRC Handbook of Chemistry and Physics. 81st Edition. CRC Press LLC, Boca Raton: FL 2000, p. 5-38] **PEER REVIEWED**

pKb = 11.02 at 20 deg C
[O'Neil, M.J. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. 13th Edition, Whitehouse Station, NJ: Merck and Co., Inc., 2001., p. 449] **PEER REVIEWED**

Prisms from water at 100 deg C /monohydrate/
[Lewis, R.J., Sr (Ed.). Hawley's Condensed Chemical Dictionary. 13th ed. New York, NY: John Wiley & Sons, Inc. 1997., p. 309] **PEER REVIEWED**

1 g is soluble in 75 mL of water /monohydrate/
[O'Neil, M.J. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. 13th Edition, Whitehouse Station, NJ: Merck and Co., Inc., 2001., p. 449] **PEER REVIEWED**

Henry's Law constant = 1.7X10-14 atm-cu m/mole at 25 deg C (est)
[US EPA; Estimation Programs Interface (EPI). ver. 3.11. U.S. EPA version for Windows. Washington, DC: U.S. EPA (2003). Available from, as of Apr 27, 2005:
http://www.epa.gov/opptintr/exposure/docs/episuite.htm] **PEER REVIEWED**

Hydroxyl radical reaction rate constant = 9.5X10-11 cu cm/molec-sec at 25 deg C (est)
[US EPA; Estimation Programs Interface (EPI). ver. 3.11. U.S. EPA version for Windows. Washington, DC: U.S. EPA (2003). Available from, as of Apr 27, 2005:
http://www.epa.gov/opptintr/exposure/docs/episuite.htm] **PEER REVIEWED**

Chemical Safety & Handling:

Disposal Methods:

SRP: The most favorable course of action is to use an alternative chemical product with less inherent propensity for occupational exposure or environmental contamination. Recycle any unused portion of the material for its approved use or return it to the manufacturer or supplier. Ultimate disposal of the chemical must consider: the material's impact on air quality; potential migration in soil or water; effects on animal, aquatic, and plant life; and conformance with environmental and public health regulations.
PEER REVIEWED

Occupational Exposure Standards:

Manufacturing/Use Information:

Major Uses:
Used in diagnosis of myocardial disfunction; dietary supplement for exercise performance
[O'Neil, M.J. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. 13th Edition, Whitehouse Station, NJ: Merck and Co., Inc., 2001., p. 449] **PEER REVIEWED**

Manufacturers:
Aceto Corp., One Hallow Lane, Lake Success, NY 11042, 516-627-6000; Production site: Piscataway, NJ 08854 /**Creatine** monohydrate/
[SRI Consulting. 2004 Directory of Chemical Producers. SRI International, Menlo Park, CA 2004., p. 521] **PEER REVIEWED**

Chattem Chemical Co., 3708 St. Elmo Ave., Chattanooga, TN, 74309. 423-822-5000; Production site: Chattanooga, TN 37409 /**Creatine** monohydrate/
[SRI Consulting. 2004 Directory of Chemical Producers. SRI International, Menlo Park, CA 2004., p. 521] **PEER REVIEWED**

Ferro Pfanstiel Laboratories, Inc., 1219 Glen Rock Ave., Waukegan, IL 60085-4039, 847-623-0370; Production site: Waukegan, IL 60085
[SRI Consulting. 2004 Directory of Chemical Producers. SRI International, Menlo Park, CA 2004., p. 521] **PEER REVIEWED**

Parish Chemical Co., 145 North Geneva Rd., Vineyard, UT 84057, 801-226-2018; Production site: Vineyard, UT 84057 /**Creatine** and creatinine/
[SRI Consulting. 2004 Directory of Chemical Producers. SRI International, Menlo Park, CA 2004., p. 521] **PEER REVIEWED**

Methods of Manufacturing:
Monosubstituted and N,N-disubstituted compounds, including guanidinocarboxylic acids (eg, **creatine**), are produced industrially by the reaction of cyanamide with the corresponding amines or aminocarboxylic acids.
[Ullmann's Encyclopedia of Industrial Chemistry. 6th ed.Vol 1: Federal Republic of Germany: Wiley-VCH Verlag GmbH & Co. 2003 to Present, p. V16 81 (2003)] **PEER REVIEWED**

General Manufacturing Information:
The literature suggests that some pharmaceutically active compounds originating from human and veterinary therapy are not eliminated completely in municipal sewage

treatment plants and are therefore discharged into receiving waters(1). Wastewater treatment processes often were not designed to remove them from the effluent(2). Selected organic waste compounds may be degrading to new and more persistent compounds that may be released instead of or in addition to the parent compound(2).
[(1) Heberer T; Tox Lett 131: 5-17 (2002) (2) Koplin DW et al; Environ Sci Toxicol 36: 1202-211 (2002)] **PEER REVIEWED**

Commercially isolated from meat extracts
[Lewis, R.J., Sr (Ed.). Hawley's Condensed Chemical Dictionary. 13th ed. New York, NY: John Wiley & Sons, Inc. 1997., p. 309] **PEER REVIEWED**

Laboratory Methods:

Clinical Laboratory Methods:

Creatine test system. (a) Identification. A **creatine** test system is a device intended to measure **creatine** (a substance synthesized in the liver and pancreas and found in biological fluids) in plasma, serum, and urine. Measurements of **creatine** are used in the diagnosis and treatment of muscle diseases and endocrine disorders including hyperthyroidism.
[21 CFR 862.1210; U.S. National Archives and Records Administration's Electronic Code of Federal Regulations. Available from, as of June 1, 2005: http://www.gpoaccess.gov/ecfr] **PEER REVIEWED**

Creatine phosphokinase/**creatine** kinase or isoenzymes test system. (a) Identification. A **creatine** phosphokinase/**creatine** kinase or isoenzymes test system is a device intended to measure the activity of the enzyme **creatine** phosphokinase or its isoenzymes (a group of enzymes with similar biological activity) in plasma and serum. Measurements of **creatine** phosphokinase and its isoenzymes are used in the diagnosis and treatment of myocardial infarction and muscle diseases such as progressive, Duchenne-type muscular dystrophy.
[21 CFR 862.1215; U.S. National Archives and Records Administration's Electronic Code of Federal Regulations. Available from, as of June 1, 2005: http://www.gpoaccess.gov/ecfr] **PEER REVIEWED**

HPLC determination in cardiac muscle.
[O'Neil, M.J. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. 13th Edition, Whitehouse Station, NJ: Merck and Co., Inc., 2001., p. 449] **PEER REVIEWED**

1H nuclear magnetic resonance (NMR) urine analysis as an effective tool to detect **creatine** supplementation. The detection limit is 10 umol/L (1.31 mg/L).
[Cartigny B et al; J Anal Toxicol 26 (6): 355-9 (2002)] **PEER REVIEWED** PubMed Abstract

Special References:

Synonyms and Identifiers:

Synonyms:
N-(Aminoiminomethyl)-N-methylglycine
PEER REVIEWED

N-amidinosarcosine
PEER REVIEWED

(alpha-methylguanido)acetic acid
PEER REVIEWED

N-methyl-N-guanylglycine
PEER REVIEWED

methylglycocyamine
PEER REVIEWED

Administrative Information:

Hazardous Substances Databank Number: 7336

Last Revision Date: 20060414

Last Review Date: Reviewed by SRP on 9/15/2005

Update History:
Complete Update on 2006-04-14, 36 fields added/edited/deleted
Created 20050407

Muscle creatine loading in men

E. HULTMAN, K. SÖDERLUND, J. A. TIMMONS, G. CEDERBLAD, AND P. L. GREENHAFF
Department of Physiology and Pharmacology, University Medical School, Queen's Medical Centre, Nottingham NG7 2UH, United Kingdom; Department of Clinical Chemistry, Huddinge University Hospital, and Department of Physiology III, Karolinska Institute, S-14186 Huddinge, Sweden

Hultman, E., K. Söderlund, J. A. Timmons, G. Cederblad, and P. L. Greenhaff. Muscle creatine loading in men. *J. Appl. Physiol.* 81(1): 232–237, 1996.—The effect of dietary creatine supplementation on skeletal muscle creatine accumulation and subsequent degradation and on urinary creatinine excretion was investigated in 31 male subjects who ingested creatine in different quantities over varying time periods. Muscle total creatine concentration increased by ~20% after 6 days of creatine supplementation at a rate of 20 g/day. This elevated concentration was maintained when supplementation was continued at a rate of 2 g/day for a further 30 days. In the absence of 2 g/day supplementation, total creatine concentration gradually declined, such that 30 days after the cessation of supplementation the concentration was no different from the presupplementation value. During this period, urinary creatinine excretion was correspondingly increased. A similar, but more gradual, 20% increase in muscle total creatine concentration was observed over a period of 28 days when supplementation was undertaken at a rate of 3 g/day. In conclusion, a rapid way to "creatine load" human skeletal muscle is to ingest 20 g of creatine for 6 days. This elevated tissue concentration can then be maintained by ingestion of 2 g/day thereafter. The ingestion of 3 g creatine/day is in the long term likely to be as effective at raising tissue levels as this higher dose.

phosphocreatine; fatigue; creatinine; skeletal muscle

STUDIES EARLIER THIS CENTURY reported that the development of fatigue during exercise in humans could be delayed by the addition of large amounts of glycine to the diet (5, 21). It was hypothesized that because glycine is a creatine (Cr) precursor, glycine ingestion would stimulate Cr biosynthesis and as a result increase muscle Cr concentration and thereby improve exercise performance. Until recently, other than these initial reports, which do not relate to Cr ingestion per se, little has been published relating to Cr ingestion and exercise performance in humans. In 1981, Sipila et al. (23) reported that in patients receiving 1.5 g Cr/day as a treatment for gyrate atrophy there was a subjective increase in strength after a 1-yr period of supplementation. Indeed, Cr ingestion was shown to reverse the type II muscle fiber atrophy associated with this disease, and one athlete in the group of patients improved his personal best record for the 100 m by 2 s (23). More recently, Cr supplementation has been shown by several laboratories to have a positive effect on short-lasting maximal exercise performance (1, 4, 9, 12, 13, 16), with only one report to date indicating that Cr ingestion has no effect on performance during this type of exercise (7). In the majority of cases, these studies have involved subjects ingesting a dose of 20 g of Cr on a daily basis for 5–6 days. This regimen was based on the work of Harris et al. (15), which was shown to result in an ~25 mmol/kg dry mass (dm) increase in muscle total Cr concentration with the between-subject variation, however, being rather large (range 2–40 mmol/kg dm). Since this initial study, no further studies have been published concerning the most appropriate procedure(s) to maximize muscle Cr uptake in men. Indeed, no information is currently available regarding the effects of different Cr doses on muscle Cr uptake or on the time course of the decline in muscle Cr after ingestion in men.

The aims of the present series of experiments were, therefore, *1*) to characterize the increase and subsequent decline in muscle Cr by using a supplementation regimen shown previously to increase muscle total Cr by ~25 mmol/kg dm (15); *2*) to determine whether, after muscle Cr concentration is elevated, muscle Cr availability could be maintained by ingesting Cr at a rate known to approximate muscle Cr degradation to creatinine (25); *3*) to assess the efficacy of relatively low-dose Cr supplementation on elevating muscle Cr concentration; and *4*) finally, to characterize the effects of Cr supplementation on urinary creatinine output. It was hoped that such a series of experiments would help clarify the procedures necessary to optimize muscle Cr retention in men.

METHODS

Thirty-one healthy men volunteered to take part in a series of four experiments, which took place over a period of 2 yr, with each subject participating in only one experiment. All subjects undertook some form of regular exercise but none was highly trained, and all were asked to maintain their normal dietary intake and to refrain from strenuous physical activity throughout each experiment. Before the experiments were begun, informed written consent was obtained from all subjects, and ethical approval was gained from the Ethical Committees of the Karolinska Institute and Nottingham University Medical School.

All subjects used in the present series of experiments were reliable individuals who had volunteered for previous experiments in our laboratories. We have no reason to believe that instructions to adhere to their normal dietary patterns and to refrain from strenuous exercise were not followed.

Experimental Groups

Group 1. This group consisted of six subjects (age 26.3 ± 2.2 yr, body mass 79.4 ± 5.8 kg). Each subject ingested 20 g Cr/day for 6 days, and muscle biopsies were obtained before supplementation (*day 0*) and on *days 7, 21,* and *35*. The dose of 20 g/day was chosen because it has previously been shown to result in an increase in muscle total Cr of ~25 mmol/kg dm (15).

Group 2. This group consisted of nine subjects (age 27.3 ± 1.8 yr, body mass 86.3 ± 2.7 kg). Each subject ingested 20 g Cr/day for 6 days and thereafter ingested Cr at a rate of 2 g/day for the next 28 days (maintenance dose). Muscle biopsy

samples were obtained before supplementation (*day 0*) and on *days 7, 21,* and *35*. The maintenance dose of 2 g/day was chosen because it has been shown that this approximates the rate of Cr degradation to creatinine (25).

Group 3. This group consisted of nine subjects (age 24.9 ± 3.6 yr, body mass 76.1 ± 2.4 kg). Each subject ingested 3 g Cr/day for 28 days, and muscle biopsy samples were obtained before supplementation (*day 0*) and on *days 15* and *29*. The dose of 3 g/day was chosen because this exceeds the reported rate of Cr degradation to creatinine (25) and represents a commonly prescribed level of supplementation.

Group 4. This group consisted of seven subjects (age 22.3 ± 1.8 yr, body mass 74.3 ± 5.4 kg). Each subject ingested 20 g of placebo (Maxijul glucose polymer; Hospital Pharmacy, Queen's Medical Centre, Nottingham, UK) for 5 days and collected a 24-h urine sample 1 and 6 days before the start of ingestion. Collections were also made on three occasions during ingestion (*days 1, 3,* and *5*) and on six occasions after Cr ingestion (*days 8, 11, 15, 18, 22,* and *25*). The same subjects then repeated the above procedures but on this occasion ingested 20 g of Cr for 5 days rather than placebo. The subjects in this experimental group ingested placebo and Cr for 5 days rather than 6 days (as in *groups 1* and *2*) because subsequent to *experiments 1* and *2*, we determined that 6 days of supplementation resulted in no further an increase in muscle total Cr than that seen after 5 days of supplementation (*day 5*: 142.6 ± 2.3 mmol/kg dm, *day 6*: 147.2 ± 4.7 mmol/kg dm; $n = 8$; $P > 0.05$; unpublished observations).

For those individuals who consumed 20 g placebo/day or 20 g Cr/day, each was requested to ingest their daily dose dissolved in ~250 ml of warm water in 5-g doses at four equally spaced intervals throughout the day. The maintenance (2 g/day) and 3 g/day doses were ingested as a single dose each day dissolved in ~250 ml of warm water. All Cr was given as Cr monohydrate in powder form (Cairn Chemicals, Chesham, UK).

Muscle and Urine Collection and Analysis

Muscle biopsy samples were on all occasions obtained from the vastus lateralis muscle of one leg by using the percutaneous needle biopsy technique (3). The biopsy sample was snap frozen in liquid nitrogen; freeze-dried; dissected free from visible connective tissue and blood; powdered; and analyzed for ATP, phosphocreatine (PCr), and free Cr (14). Total Cr was calculated as the sum of PCr and free Cr. All individual PCr and free Cr values were corrected by using ATP, which is known to show very little variation between individuals and between muscle sampling sites (14, 15). Twenty-four-hour urine samples were collected in containers to which sodium hypochlorite (1% wt/vol) had been added. After collection, all samples were measured for urinary volume and mixed thoroughly, and an aliquot was removed and stored frozen at −80°C. Samples were later analyzed for urinary creatinine concentration by using high-performance liquid chromatography (8).

Calculations and Statistical Analysis

The net uptake or release of muscle Cr (mmol) was calculated from the change in muscle total Cr (mmol/kg wet muscle) × estimated muscle mass (40% of body mass). Statistical analysis was performed by using analysis of variance for repeated measures. When a significant F value was achieved, a Tukey post hoc test was applied and significance was accepted at the 5% level. All values shown in the text, Tables 1 and 2, and Figs. 1–3 are means ± SE.

RESULTS

Muscle Metabolite Changes

Groups 1 and 2. No change in ATP concentration was observed over the course of the study in either experimental group (Table 1).

Presupplementation muscle total Cr concentration did not significantly differ between *groups 1* and *2* (123.4 ± 3.0 mmol/kg dm and 119.5 ± 2.5 mmol/kg dm, respectively). Both *groups 1* and *2* demonstrated an average increase in muscle total Cr concentration of ~23 mmol/kg dm after 6 days of supplementation (Fig. 1, *A* and *B*; $P < 0.05$). This corresponded to ~20 g of Cr retention, which represented ~17% of the total amount ingested.

In *group 1*, muscle total Cr declined toward the presupplementation concentration at a rate of ~0.43 mmol·kg dm^{-1}·day^{-1} over the after 28 days (Fig. 1*A*). By *day 21* muscle total Cr had decreased by ~6 mmol/kg dm to 139.2 ± 6.3 mmol/kg dm, and by *day 35* it did not significantly differ from the presupplementation concentration. In *group 2*, after the initial 6 days of supplementation, muscle total Cr concentration remained constant for the remaining 28 days when Cr was ingested at the rate of 2 g/day (Fig. 1*B*).

In both *groups 1* and *2*, the increase in muscle total Cr concentration during the initial 6 days was composed mainly of a change in free Cr, which increased by ~16 mmol/kg dm in each group (Table 1; $P < 0.05$). The mean muscle PCr concentration increased by ~8 mmol/kg dm over the same period, but this failed to reach statistical significance (Table 1). Indeed, muscle PCr concentrations were not significantly different between time points within each group during the 35-day experimental period. However, when data from experimental *groups 1* and *2* were combined, the increase in PCr concentration observed after 6 days of supplementation was significant ($n = 15$; $P < 0.05$). It was also noted that the PCr-to-ATP ratio (PCr/ATP) was significantly increased after 6 days of supplementation when data from both groups were combined (pre-

Table 1. *ATP, PCr, and free Cr concentrations recorded over 35 days in experimental groups 1 and 2*

	ATP	PCr	Free Cr
		Group 1	
Day 0	25.63 ± 1.12	80.36 ± 3.78	43.01 ± 2.65
Day 7	23.25 ± 1.12	87.88 ± 3.05	57.24 ± 4.00*
Day 21	23.60 ± 0.91	88.63 ± 4.24	50.62 ± 2.55
Day 35	24.59 ± 0.70	86.77 ± 4.11	45.62 ± 2.24
		Group 2	
Day 0	23.77 ± 0.52	76.58 ± 1.35	42.87 ± 2.43
Day 7	23.22 ± 0.66	83.78 ± 3.45	59.85 ± 2.04*
Day 21	22.81 ± 0.46	84.51 ± 3.24	59.77 ± 3.13*
Day 35	24.40 ± 0.48	84.75 ± 2.13	58.32 ± 2.15*

Values are means ± SE for 6 subjects in *group 1* and 9 subjects in *group 2*. Cr, creatine; PCr, phosphocreatine. *Significantly different from *day 0*, $P < 0.05$. *Group 1* consumed Cr at a rate of 20 g/day for the initial 6 days. *Group 2* consumed Cr at a rate of 20 g/day for 6 days followed by 2 g/day for the remainder of the experiment.

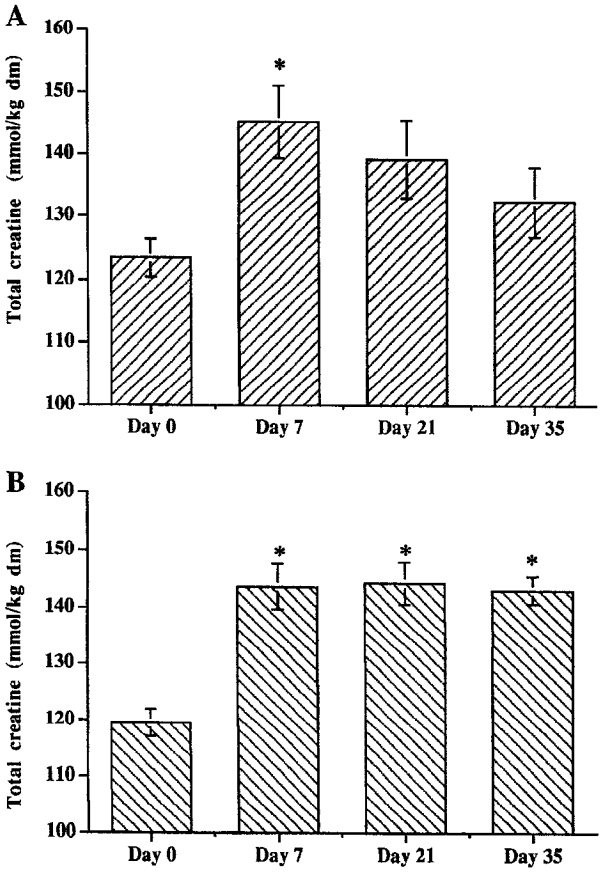

Fig. 1. *A*: muscle total creatine concentration in experimental *group 1* ($n = 6$). All subjects ingested 20 g of creatine for 6 consecutive days, and muscle biopsy samples were obtained before ingestion (*day 0*) and on *days 7, 21*, and *35*. *B*: muscle total creatine concentration in experimental *group 2* ($n = 9$). All subjects ingested 20 g of creatine for 6 consecutive days and thereafter ingested creatine at a rate of 2 g/day for the next 28 days. Muscle biopsy samples were taken before ingestion (*day 0*) and on *days 7, 21*, and *35*. Values are means ± SE. dm, Dry mass. *Significantly different from *day 0*, $P < 0.05$.

supplementation: 3.2 ± 0.1 mmol/kg dm, *day 7*: 3.7 ± 0.2 mmol/kg dm; $P < 0.01$; $n = 15$) and was still elevated in both groups at *day 21* (*group 1*: 3.7 ± 0.2 mmol/kg dm, $P < 0.05$; *group 2*: 3.8 ± 0.2 mmol/kg dm, $P < 0.05$). However, by *day 35*, PCr/ATP did not significantly differ from the presupplementation ratio in either group (*group 1*: 3.5 ± 0.1 mmol/kg dm, *group 2*: 3.5 ± 0.1 mmol/kg dm).

Group 3. No change in ATP concentration was observed in experimental *group 3* over the course of the study (Table 2).

Table 2. *ATP, PCr, free Cr, and total Cr concentrations recorded over 28 days in experimental group 3*

	ATP	PCr	Free Cr	Total Cr
Day 0	23.63 ± 0.70	76.89 ± 2.64	44.87 ± 3.64	121.76 ± 3.35
Day 14	23.51 ± 0.76	83.46 ± 2.05‡	53.08 ± 2.33†	136.54 ± 3.14†
Day 29	23.13 ± 0.53	86.00 ± 1.69‡	56.05 ± 2.55†	142.04 ± 3.18†

Values are means ± SE for 9 subjects in *group 3*. *Group 3* consumed Cr at a rate of 3 g/day for the initial 28 days. Significantly different from *day 0*: †$P < 0.01$; ‡$P < 0.001$.

Muscle total Cr concentration increased by ~15 mmol/kg dm during the initial 14 days of supplementation ($P < 0.01$), which was less than the increases observed in experimental *groups 1* and *2* after 6 days of supplementation. After 28 days of supplementation, muscle total Cr concentration had increased by a further 6 mmol/kg dm ($P = 0.06$), such that uptake over the whole experimental period was ~20 mmol/kg dm ($P < 0.01$). This increase was not significantly different from the increases observed in experimental *groups 1* and *2* after 6 days of supplementation. The uptake of Cr over the initial 14 days of supplementation was equivalent to ~13 g, which was ~30% of the total amount of Cr ingested. During the final 14 days, Cr uptake was ~5 g, which was ~12% of the total amount of Cr ingested. Similar to *groups 1* and *2*, the increase in total Cr over the 28 days of supplementation was composed mainly of an increase in free Cr (Table 2).

PCr/ATP did not significantly increase in *group 3* during the 28-day supplementation period (*day 0*: 3.3 ± 0.1 mmol/kg dm, *day 14*: 3.5 ± 0.1 mmol/kg dm, and *day 28*: 3.7 ± 0.1 mmol/kg dm). However, the numerical increases in PCr/ATP were similar to those observed in both *groups 1* and *2* after similar increases in muscle total Cr.

Urinary Creatinine

The daily urinary creatinine output before, during, and after placebo and Cr ingestion in experimental *group 4* is shown in Fig. 2. A large amount of variation was seen between subjects when urinary creatinine output is compared, but over the final 20 days of the study there was on average a 2.8 mmol/day greater excretion of creatinine after Cr ingestion when com-

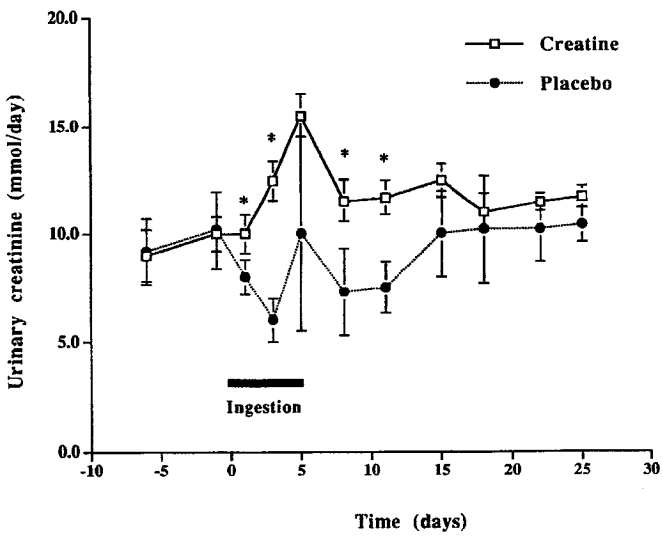

Fig. 2. Urinary creatinine output in experimental *group 4* before and after placebo and creatine supplementation. Experimental *group 4* ($n = 7$) ingested 20 g of glucose polymer (placebo) for 5 days. All subjects undertook a 24-h urine collection on 2 occasions before ingestion, on 3 occasions during ingestion, and on 6 occasions over 20 days after ingestion. All subjects then repeated the above procedures, but on this occasion ingested creatine rather than placebo. Values are means ± SE. *Significant difference between corresponding creatine and placebo time points, $P < 0.05$.

pared with placebo ingestion ($P < 0.05$). Figure 3 shows urinary volume for subjects in *group 4*. Similar to urinary creatinine output, there was a large amount of variation between subjects. However, Fig. 3 clearly indicates Cr ingestion markedly reduced urinary volume during the initial days of Cr supplementation ($P < 0.001$). It should be noted that daily urinary volume after Cr ingestion returned to normal levels before urinary creatinine output was significantly elevated above that observed after placebo ingestion.

DISCUSSION

Cr was first identified in meat extract in 1835 by Chevreul (see Ref. 20). Even in the early part of this century, there was already literature pointing to an important function for Cr in muscle contraction, the knowledge of its specific distribution, and its absence from normal urine, leading to the realization that it was not merely a waste product of metabolism (20). This realization was confirmed when Chanutin (6) observed that Cr administration resulted in a major portion of the compound being retained by the body. From these early studies, Cr retention in the body pool was thought to be much greater during the initial stages of administration (2, 6). More recently, in vitro work has demonstrated that Cr transport is sodium dependent (18) and occurs via a discrete Cr transporter protein in mammalian skeletal muscle (22). Furthermore, with the application of the muscle biopsy technique, it has recently become clear that the ingestion of 20 g of Cr each day for 5–6 days (4 × 5-g doses) can, on average, result in an ~20% increase in muscle total Cr concentration in humans, of which ~30% is in the form of PCr (11, 15). In agreement with earlier work (2, 6), it was demonstrated that the majority of tissue Cr uptake occurred during the initial days of supplementation, with close to 30% of the administered dose being retained during the initial 2 days of supplementation compared with 15% from *days 2–4*. Furthermore, when submaximal exercise was performed during the period of supplementation, muscle uptake appeared to be increased by a further 10% (15). However, these studies did not characterize the time course of muscle total Cr increase and subsequent decline or the efficacy of high- and low-dose Cr ingestion.

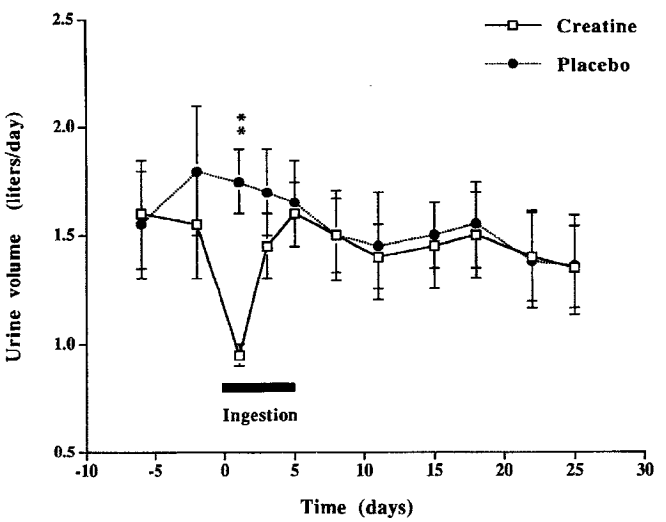

Fig. 3. Urinary volume in experimental *group 4* before and after placebo and creatine supplementation. Experimental *group 4* ($n = 7$) ingested 20 g of glucose polymer (placebo) for 5 days. All subjects undertook a 24-h urine collection on 2 occasions before ingestion, on 3 occasions during ingestion, and on 6 occasions over 20 days after ingestion. All subjects then repeated the above procedures but on this occasion ingested creatine rather than placebo. Values are means ± SE. **Significant difference between corresponding creatine and placebo time points ($P < 0.01$).

The mean increase in muscle total Cr concentration observed in the present series of studies, when Cr was ingested at a rate of 20 g/day for 6 days, was on all occasions >20 mmol/kg dm and was similar to that reported earlier (11, 15). When Cr was ingested at a lower dose (3 g/day), the rate of muscle Cr uptake was correspondingly lower. However, after 28 days of supplementation at this lower rate, no difference in muscle total Cr concentration was observed between regimens. This demonstrates that Cr ingestion at a rate of 3 g/day will in the long term be just as successful at increasing muscle total Cr as will the 20 g/day regime. However, the present study clearly demonstrates that a more rapid way to increase the muscle Cr store is to ingest a dose close to 0.3 g/kg body mass for 6 days. To maintain this high muscle Cr store, it is not necessary to maintain a high dietary Cr intake because a dose rate close to 0.03 g/kg body mass will maintain tissue levels (Fig. 1*B*). The present series of findings are generally in agreement with in vitro experiments that have demonstrated that Cr entry into skeletal muscle is initially dependent on the extracellular Cr concentration but is subsequently downregulated in the presence of elevated extracellular and intracellular Cr concentrations (18, 19).

Figure 1*A* shows that there was a continuous loss of Cr from muscle after 6 days of Cr loading (20 g/day). This loss was matched by a parallel increase in creatinine formation and excretion (Fig. 2). For example, during the initial 14 days after Cr ingestion, the decline in muscle Cr was 40 mmol (assuming a muscle mass of 28 kg wet wt), which was very close to the 39-mmol increase in creatinine excretion noted in *group 4* over the same period. This suggests that the rate of creatinine formation is directly proportional to the muscle Cr concentration and indicates that the endogenous production of Cr may not be inhibited after Cr ingestion.

Data from *groups 1* and *2* demonstrate that ~70% of the increase in total Cr after Cr ingestion was in the form of free Cr, the remainder being phosphorylated. Initially, on the cessation of Cr ingestion (*group 1*), muscle PCr remained relatively constant and free Cr accounted for the majority of the decline seen in total Cr. This was reflected in PCr/ATP, which was increased after 6 days of 20 g/day Cr supplementation and remained elevated until a substantial fall in muscle total Cr was observed (*day 35*). This finding also lends some support to the suggestion that muscle phosphate uptake may occur in conjunction with Cr uptake (G. K. Radda, personal communication) The decline in PCr/

ATP in both *groups 1* and *2* after 21 days is difficult to explain, especially because there was no decline in muscle total Cr concentration in *group 2*. Additionally, there was no significant change in PCr/ATP in *group 3* throughout the 28 days of 3 g/day supplementation, despite an increase in muscle total Cr concentration similar to those observed in *groups 1* and *2*. This perhaps suggests that there may be some acute metabolic response associated with a rapid increase in muscle total Cr that is not observed when muscle Cr concentration is increased more slowly.

Previous studies have demonstrated that Cr ingestion at a rate of 20 g/day for a period of 5–6 days can significantly improve maximal exercise performance (1, 4, 12, 13, 16) and the rate of PCr resynthesis during recovery (11). We have previously reported that this improvement in exercise performance may have been achieved as a result of an increase in resting preexercise PCr concentration (12) and/or as a consequence of a free Cr-mediated increase in PCr resynthesis rate during and after exercise (11). However, given the small magnitude of the increase in PCr in relation to Cr in the present series of experiments, it would appear that the previously reported ergogenic effect of Cr ingestion was principally mediated by the latter mechanism. In support of this suggestion, it has been demonstrated that both the improvement of exercise performance and the enhancement of postexercise PCr resynthesis after Cr ingestion are positively associated with the extent of muscle total Cr retention during supplementation (10, 11).

Several studies have reported that 5–6 days of Cr ingestion at the rate of 20 g/day will result in a body mass increase of 0.5–1.0 kg (1, 11, 24). It has also recently been demonstrated that 28 days of Cr ingestion at a rate of 20 g/day resulted in a 1.7-kg increase in body mass (9), which was greater than the increases previously reported over a 5- to 6-day period (1, 11, 24). Earnest et al. (9) attributed this body mass increase to an increase in fat-free mass. Whether this was composed of water or protein remains to be demonstrated. However, the 0.6-liter decline in urinary volume that occurred at the onset of Cr ingestion in subjects in experimental *group 4* of the present study (Fig. 3) suggests the increase in body mass during acute Cr feeding is likely to be attributable to body water retention. It should also be noted that the time course of urinary volume changes paralleled the time course of muscle Cr uptake documented by Harris et al. (15).

In conclusion, it would appear that a rapid way to "Cr load" skeletal muscle in humans is to ingest 20 g of Cr for 6 days. The elevation in tissue Cr concentration achieved can then be maintained by ingesting 2 g/day thereafter. Alternatively, the ingestion of 3 g of Cr/day over a minimum period of 4 wk is likely to be as effective at raising tissue levels as the higher dose regimen, albeit at a slower rate. On the basis of the results of the present series of studies, it would appear that an effective way to obtain immediate and sustained performance benefits from Cr ingestion may be to use a loading dose of 0.3 g·kg body mass^{-1}·day^{-1} for a period of 5–6 days, followed by a maintenance dose of 0.03 g·kg body mass^{-1}·day^{-1} thereafter.

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GAMT joins the p53 network

Branching into metabolism

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The p53 protein functions to prevent tumor development by restricting proliferation, motility and survival of abnormal or stressed cells. In addition to well-established roles, recent discoveries indicate a role for p53 in the regulation of pathways involved in energy metabolism. The metabolic functions of p53 can inhibit the shift to glycolysis that is characteristically seen in cancer cells, while favoring the energy production by mitochondrial oxidative phosphorylation. Identification of guanidinoacetate methyltransferase (GAMT) as a new p53 target connects p53 to creatine metabolism critical in the regulation of ATP homeostasis. The involvement of GAMT in both genotoxic and metabolic stress-induced apoptosis, as well as the requirement of p53-dependent upregulation of GAMT in glucose starvation-mediated fatty acid oxidation (FAO), demonstrate a further role of p53 in coordinating stress response with changes in cellular metabolism. Such activities of p53 would help to bring a better understanding of how cancer cells acquire unique metabolic features to maintain their own survival and proliferation, and might provide interesting clues toward the development of novel therapies.

Key words: p53, cancer cell metabolism, GAMT, creatine metabolism, fatty acid oxidation

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Introduction

Since its discovery 30 years ago, the p53 protein has emerged as a key tumor suppressor protein, and beyond doubt, a crucial player in cancer biology. p53 invokes its tumor-suppressive ability by acting as a mediator of various kinds of stress, such as DNA damage, oxidative stress and oncogene activation.[1] Through its activity as a transcription factor, p53 regulates the expression of various target genes to prevent tumor development, mainly by inducing cell cycle arrest and DNA repair or triggering cell death and senescence to maintain genomic stability.[2-6] Under mild or transient stress conditions, activated p53 targets several genes involved in cell cycle arrest and DNA repair to stop cells from proliferating and allow repair of any damaged DNA, preventing potentially oncogenic mutations from being passed on to the daughter cells. However, when stress-induced DNA damage is too severe to be reparable, p53 initiates programmed cell death/apoptosis and cellular senescence to eliminate or permanently arrest cells, respectively, that may have acquired irreparable and potentially oncogenic mutations. Relevantly, the human p53 gene (*TP53*) is frequently mutated or inactivated in more than 50% of human cancers of different types.[7] Furthermore, mice with a p53 gene (*Trp53*) deletion can develop normally but develop cancer before the age of 6 months.[8] Thus, the importance of p53 in the inhibition of tumor development is indisputable; however, the function of p53 is far from simplicity. To date, emerging evidence indicates that p53 is involved in numerous pathways and is capable of much broader cellular functions, ranging from fertility, development and aging to energy metabolism and autophagy.[9-14] Furthermore, it is clear that the activity of p53 by modulating metabolic pathways

will have consequences beyond cancer, influencing various other aspects of disease and longevity.

Cancer Cell Metabolism

Rapid cell growth and proliferation are representative features of tumor cells. Consequently, tumor cells need ample amount of energy to generate macromolecules (DNA, RNA, proteins and lipids) necessary for cell proliferation. To fulfill such demand for energy, tumor cells undergo modifications in cellular metabolism and metabolic adaptation to support its enhanced cell growth and proliferation and to survive periods of metabolic stress and maintain viability. Among various nutrients, glucose is the primary energy source for most normal cells. Under conditions of normal oxygen level, glucose is metabolized via mitochondrial oxidative phosphorylation to efficiently generate 32 molecules of ATP per one molecule of glucose. However, when oxygen level is low, mitochondrial function is suppressed and normal cells undergo anaerobic glycolysis to produce only a fraction of the maximum energy from glucose (two molecules of ATP per one molecule of glucose). Thus, normal cells would not use this less efficient pathway to produce energy under aerobic conditions. On the contrary, tumor cells, which require substantial amount of energy, preferentially utilize the less efficient glycolytic pathway even though sufficient level of oxygen is available (also known as "aerobic glycolysis"). This striking discovery was first documented by Otto Warburg in the 1920s when he observed that liver cancer cells, compared to normal liver cells, displayed an increase in glycolytic activity despite the presence of oxygen.[15] He further hypothesized that this increase in aerobic glycolysis is due to mitochondrial dysfunction and may be the prime cause of cancer.[16] Whether this metabolic shift in glucose metabolism from oxidative phosphorylation to aerobic glycolysis is the origin of cancer or a consequence of tumorigenesis, this phenomenon, termed the "Warburg effect," has been reported in most cancers and is recognized as a key metabolic hallmark of virtually all cancer cells.[17,18] This metabolic change is now widely used in diagnosing human solid tumors using fluorodeoxyglucose positron emission tomography (FDG-PET) to detect the much higher uptake of glucose by the tumor than the adjacent normal tissues. In addition, an increasing understanding of the molecular mechanisms that control metabolism highlights the realization that metabolic transformation can have an essential role in maintaining tumorigenic state.

Role of p53 between Glycolytic and Respiratory Pathways

Recent studies have demonstrated the ability of p53 in the regulation of both glycolysis and oxidative phosphorylation, consequently contributing to prevent the increase in glycolysis that is characteristic of cancers. p53 can reduce glucose uptake into the cells through inhibiting the expression of glucose transporters GLUT1 and GLUT4 as the first defense.[19] Glucose uptake is further limited by p53's regulation of NFκB pathway.[20] Expression of p53 can restrict the activity of IκB kinase-α (IKKα) and IKKβ, thereby leading to a reduction in NFκB activity and decreased expression of GLUT3. p53 can also repress the levels of PGM (phosphoglycerate mutase), which acts at the later stages of the glycolytic cascade,[21] and TIGAR (TP53-induced glycolysis and apoptosis regulator), which functions to direct glucose to an alternative pathway, the pentose phosphate pathway (PPP).[22] Loss of p53 is associated with increased PGM and decreased TIGAR expression, which can enhance glycolysis and the Warburg effect.

The modulation of glycolytic rate by p53 is paralleled by the ability of p53 to help maintain mitochondrial function and promote oxidative phosphorylation. SCO2 (synthesis of cytochrome oxidase 2), a target gene of p53, regulates the cytochrome c oxidase complex, which is essential for mitochondrial respiration and utilization of oxygen to produce energy (ATP).[23] Another p53 target gene AIF (apoptosis-inducing factor) plays a role in regulating various cell death pathways and, as an oxidoreductase, is a key factor in maintaining the integrity of complex 1 in the electron transport chain.[24] Thus, cells that lack functional p53 show lower oxygen consumption by mitochondrial respiration and a shift to glycolysis for the production of energy.

p53 has been further implicated in metabolic control through its communication with two key regulatory factors, AMPK (AMP-activated protein kinase) and mTOR (mammalian target of rapamycin).[25,26] Reduced nutrient or energy levels result in the activation of AMPK and failure to stimulate the AKT-mTOR pathway, both of which can induce p53, leading to enhanced macroautophagy and fatty acid oxidation.[27,28] The aforementioned p53 regulation of energy metabolism is merely a subset among the various aspects of metabolism that p53 can regulate, and without a doubt, this complex network of p53 will have more additions.

p53 and Creatine Metabolism

The creatine-phosphocreatine system plays an important role in phosphate-bound energy storage and transmission. The reversible phosphorylation of creatine by creatine kinase with ATP/ADP provides a high-energy phosphate buffering system.[29] This system is essential in cells and tissues with high and fluctuating energy demands. Creatine is synthesized in a two-step mechanism by two enzymes: AGAT (arginine:glycine amidinotransferase) and GAMT (guanidinoacetate methyltransferase). AGAT, primarily expressed in the kidney and pancreas, catalyzes the first biosynthetic step of creatine by taking glycine and arginine to produce ornithine and GAA (guanidinoacetate). Subsequently, GAA enters the blood stream to reach the liver where it is methylated by GAMT to yield creatine. Creatine is then exported back into the blood stream to be taken up by tissues requiring creatine, such as muscle, brain and heart, through active creatine transporters. Loss of GAMT causes a creatine deficiency syndrome, first described in 1994, that is characterized by developmental delay, mental retardation, neurological and motor dysfunction.[30-34] Aside from the more commonly known function and disorders of creatine metabolism, other roles of creatine metabolism exist. Since creatine

metabolism is intimately connected with ATP homeostasis and tumor cells have high demand for ATP, the role of creatine metabolism in cancer cells is conceivable and may be of importance. In fact, the association between creatine metabolism and cancer has long been reported in the literature.[35-42] However, when levels of creatine content and creatine kinase activity were examined in malignant cells and tumor-bearing animals, the results are somewhat inconsistent. Some reports show increased creatine content and elevated creatine kinase activity in various human carcinoma tissues,[35,36,38,42] while some show downregulation of the creatine kinase system in malignant tissues and tumor-bearing mice.[37,39,41,43] It is possible that the specific role of creatine shuttle in cancer is tissue and isoform specific, as several tissue-related isoforms of creatine kinase exist: muscle, mitochondrial and brain creatine kinase.

In connection with p53 and creatine metabolic pathway, mouse p53 has been reported to repress the expression of rat brain creatine kinase but activate the rat muscle creatine kinase gene, although it is unclear of how p53 and creatine kinase function in cell metabolism.[44] Recently, GAMT was identified as a novel p53 target, demonstrating another metabolic pathway, namely creatine metabolism, by which p53 can control to adapt to metabolic stress.[45] Overexpression of p53 or inducing p53 by etoposide treatment leads to an increase in creatine level that is reduced upon ablation of GAMT. Moreover, depletion of creatine by treating cells with creatine circuit inhibitor produces less etoposide-mediated apoptosis. In response to glucose deprivation, GAMT is induced in a p53-dependent manner, and levels of GAMT and creatine are increased in several tissues of nutrient-deprived p53 wild-type mice while remaining unchanged in the same tissues of p53 null mice. GAMT ablation also reduces glucose depletion-induced apoptosis, demonstrating that GAMT is not only involved in p53-dependent apoptosis in response to genotoxic stress but is important for apoptosis induced by nutrient starvation. It is well established that increased level of reactive oxygen species (ROS) can initiate apoptotic pathway.[46,47] Therefore, etoposide treated cells result in an increase in intracellular ROS level that is inhibited by creatine circuit inhibitor, and creatine treated cells produced an increase in intracellular ROS level. These findings suggest a new role for GAMT and creatine metabolism in p53-dependent apoptosis. Since some reports have also shown anticancer effects of creatine by leading to the increased formation of nitric oxide,[48] emerging possibility implicates that p53-creatine metabolic pathway might function as tumor suppressing mechanism, although further examinations are required.

Altered Lipid Metabolism in Cancer Cells

Although the Warburg effect has been recognized for 90 years, alterations in lipid metabolism are less well appreciated. Several studies reveal that many tumors have high rates of de novo fatty acid biosynthesis regardless of the concentration of extra cellular lipids, which primarily reflects dietary fats.[49-51] Fatty acid synthase (FASN) and the enzymatic activity of ATP citrate lyase are increased to support the synthesis of fatty acids.[52] FASN is a lipogenic enzyme which catalyzes the de novo synthesis of long-chain fatty acids from acetyl-CoA, malonyl-CoA and NADPH precursors and is overexpressed in several human cancers.[53] It is reported that PI3K/Akt pathway stimulates fatty acid synthesis via activation of ATP citrate lyase and inhibition of fatty acid oxidation (FAO) via reduced expression of CPT1 (carnitine palmitoyltransferase 1). CPT1, which catalyzes the transport of long-chain fatty acids into mitochondria for FAO, is recently reported to be decreased in human cancer specimens.[54] Furthermore, mouse mammary carcinoma models and human primary breast cancer often show diminished expression of DecR1 (2,4-dienoyl-coenzyme A reductase), another enzyme involved in FAO. More importantly as well, ectopic expression of DecR1 reduced tumor growth and decreased de novo fatty acid synthesis,[55] providing the therapeutic potential of targeting tumor cell fatty acid synthesis.

The tumor microenvironment is spatially and temporally heterogeneous, containing region of low oxygen and low glucose. When glucose is not available, FAO is reported to be the first alternate pathway used by most tissues to generate energy.[56,57] Our results show that increased FAO ensues and that this requires p53 and GAMT in response to glucose deprivation.[45] Further examinations reveal that creatine increases phosphorylation of AMPK and ACC (acetyl-CoA carboxylase), indicating that FAO has been switched on. Moreover, similar evidence came from the observation that increased FAO in liver occurs upon starvation of wild-type but not p53 deficient animals, and p53 deficient animals have generally lower levels of liver FAO than their wild-type counterparts, indicating p53 in energy maintenance by FAO pathway. FAO connects to Krebs cycle by converting Acyl-CoA to Acetyl-CoA and contributes to maintaining oxidative phosphorylation, and increased FAO can inhibit glycolysis.[58] Thus, p53-GAMT regulation of FAO may function to keep the balance between glycolytic and respiratory pathway to oppose the metabolic shift (Warburg effect) in tumorigenic state (**Fig. 1**).

Concluding Remarks

In summary, p53 can communicate with creatine biosynthetic and FAO pathways through its target gene GAMT to regulate energy metabolism. It still remains a challenge to understand when and how each of the p53 metabolic target genes or outcomes is all coordinated. Metabolic changes are emerging as key contributors to malignant progression and most cancer cells show the characteristic increase in aerobic glycolysis known as the Warburg effect. Better understanding of p53 and its targets in energy metabolism may hold the key to effective therapeutic approaches against cancer and metabolism-related diseases.

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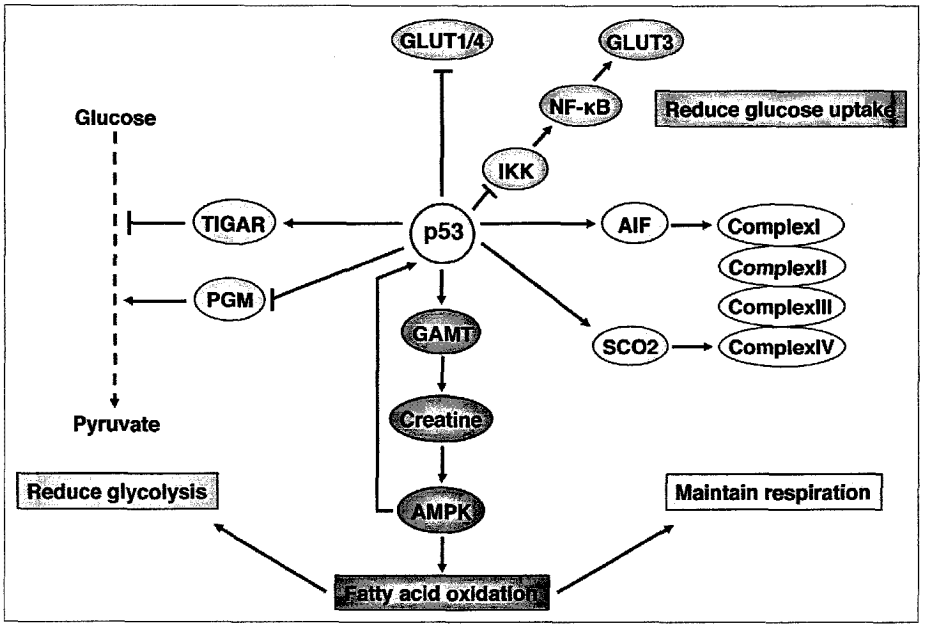

Figure 1. Regulation of energy metabolism by p53. Several functions of p53 slow the flux through the glycolytic pathway and promote oxidative phosphorylation, thereby opposing the metabolic shift (Warburg effect) by which cancer cells often use glycolysis for energy production. p53-GAMT regulation of FAO also joins this network as a balancer between glycolytic and respiratory pathway. GLUT, glucose transporter; IKK, IκB kinase; NFκB, nuclear factor-κB; TIGAR, TP53-induced glycolysis and apoptosis regulator; PGM, phosphoglycerate mutase; AIF, apoptosis-inducing factor; SCO2, synthesis of cytochrome oxidase 2; GAMT, guanidinoacetate methyltransferase; AMPK, AMP-activated protein kinase.

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